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The Source of Proline for Casein Biosynthesis

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(*Received* 28 *February* 1958)

Previous work has shown that the mammary gland takes the free essential amino acids from the blood stream to provide the essential amino acids of casein (Barry, 1952; Campbell & Work, 1952; Askonas, Campbell, Godin & Work, 1955); that it takes free glutamine and glutamic acid to provide the glutamine and glutamic acid respectively of casein (Barry, 1956; Sheldon-Peters & Barry, 1956); and that it takes free asparagine to provide asparagine, but not aspartic acid, residues of casein (Sansom & Barry, 1958).

Experiments described in this paper show that the mammary gland of the goat takes free proline from the blood to provide at least 50% of the proline residues of casein. It is calculated from this and previous work that at least half of the residues of non-essential amino acids in casein are not synthesized within the mammary gland, but come directly from the blood stream.

EXPERIMENTAL

L-[^{14}C]*Proline* (*uniformly labelled*). This was bought from the Radiochemical Centre, Amersham, Bucks.

Experiments on goat. The experiments were done on a goat which was giving about 1 l. of milk/day, and was in good health. Compounds were injected, and blood and milk samples taken, as described by Barry (1952).

Isolation of free proline of blood plasma. Blood plasma (40 ml.) was dialysed overnight against distilled water (200 ml.) on a shaking machine, and the diffusate was freeze-dried. The residue was dissolved in a little water, brought to pH 3 and eluted from a column of Dowex-50 (100 cm. × 1·15 cm.) as described by Moore & Stein (1951), but at room temperature. The proline fractions were combined, their proline content was accurately determined by the quantitative ninhydrin method (Moore & Stein, 1948) and a known weight of inactive proline added. Salts were then removed (Sheldon-Peters & Barry, 1956) and the solution was evaporated to give a crystalline sample of proline. From the specific activity of this sample, and the dilution by inactive proline, the specific activity of the free proline of plasma was calculated.

Isolation of proline from casein. Casein was isolated and hydrolysed as before (Barry, 1952), and a sample of its proline was isolated for ^{14}C determination in the same way as the free proline of blood.

Isolation of glutamic acid from casein. The glutamic acid in an acid hydrolysate of casein was isolated by elution from a column of Dowex-1 (acetate form; 20 cm. × 1·6 cm.) with 0·5N-acetic acid (Hirs, Moore & Stein, 1954). Evaporation of the fractions containing glutamic acid gave a crystalline sample.

Determination of ^{14}C *specific activities.* The crystals of proline or glutamic acid were packed in aluminium cups (1·5 cm. diameter) to give samples of infinite thickness, and put in a standard position under the window of a Geiger counter. They were counted to give less than 3% probable error, and the counts were converted into specific activities with a factor determined previously.

RESULTS

Goat's milk contains about 3% of casein, in which the amino acid concentrations are very similar to those in cow's casein (Davis & Macdonald, 1953). On hydrolysis, 100 g. of cow's casein yields 11·3 g. of proline (McMeekin & Polis, 1949). Human blood plasma contains about 2 mg. of free proline/100 ml. (Stein & Moore, 1954), and the concentrations in the plasma of the goat were found to be similar. The proline of casein might therefore come directly from the free proline of blood, or from that of plasma proteins, or might be synthesized within the mammary gland from other compounds absorbed from the blood.

The experiment shown in Fig. 1 was designed to find the proportion of the proline of casein which is derived from the free proline of blood. [^{14}C]Proline was injected intravenously into a goat, thus labelling the free proline of its blood, and blood and milk samples were taken at intervals after the injection. It can be seen that the specific activity of the proline of casein rapidly became comparable with that of the free proline of blood plasma. The curve *A* passes through each value for the specific activity of casein proline, in the last three milk samples, at a time half-way through the period in which the milk was secreted, and shows the specific activity of casein proline, at various times, in milk which has just reached a point in the mammary gland from which it can be drawn off by milking. It can be seen that after 2¼ hr. the specific activity of casein proline was always equal to that of the free proline of blood plasma 1–2 hr. earlier.

In an experiment of this type, the area under the specific activity–time histogram of a component of milk, divided by the area under the specific-activity curve of the labelled component of blood, gives the proportion of the component of milk

which is derived from that of blood, provided that only one constituent of the blood has significant radioactivity (Barry, 1956). In this experiment, significant radioactivity would be gained by any component of the blood only if the component were continually and rapidly synthesized from free proline. It can be predicted from previous studies (Barry, 1952, 1956; Sansom & Barry, 1958) that the proline of plasma proteins would have gained little activity during this experiment, and that the area under a curve showing the specific activity of the proline of plasma proteins would have been less than 5% of that under the curve for the free proline of plasma. The only other component of blood, a significant part of which might be continually and rapidly synthesized from free proline, is the free glutamic acid (Stetten, 1955). In a previous similar experiment (Barry, 1956) it was shown that the area under the specific-activity curve for the free glutamic acid of blood equals the area under the specific-activity histogram for the glutamic acid of casein. In the present experiment the activity of the glutamic acid in an acid hydrolysate of each casein sample was measured, and the results are shown in Table 1. It can be calculated that the area under the specific-activity histogram is only 3·3 units. Although the glutamic acid in the acid hydrolysate came from both glutamic acid and glutamine residues, it may be concluded that the area under the specific-activity curve for the free glutamic acid of blood was also about 3·3 units, and was therefore not significant compared with the area under the curve for the free proline. It is clear that an insignificant portion of the glutamic acid of blood is synthesized from proline, although proline is apparently metabolized via glutamic acid (Stetten, 1955).

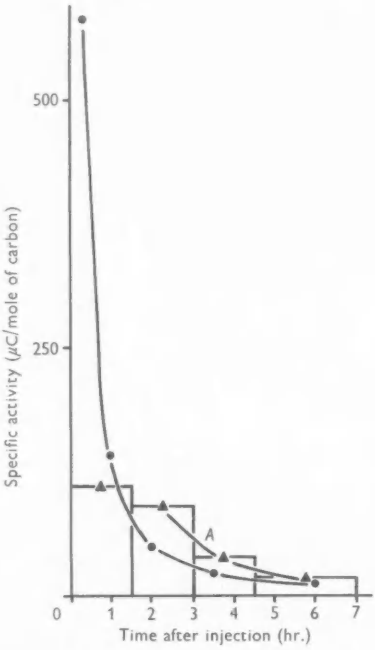

Fig. 1. Use of proline for synthesis of casein. L-(^{14}C)-Proline (0·32 mg.; 25 μc) was injected intravenously into a lactating goat. The goat was milked immediately before injection, and blood and milk samples were taken at intervals up to 7 hr. after. ●, Free proline of plasma; ▲, curve *A*, the significance of which is described in the text. The histograms show the specific activities of casein proline in each milk sample, the width of each rectangle occupying the time during which the sample was secreted.

Table 1. *Specific activity of glutamic acid in acid hydrolysates of casein samples*

Samples were taken at intervals after injecting L-[^{14}C]-proline (0·32 mg.; 25 μc) into a lactating goat.

Time of sampling (hr.) ...	1·5	3·0	4·5	7·0
Specific activity (μc/mole of carbon)	0·36	0·84	0·51	0·31

In Fig. 1 the area under the casein-proline histogram up to 7 hr. is 408 units, and that under the plasma-proline curve (if extrapolated back to the ordinate) up to 5·5 hr. is 580 units. (The time difference allows for the lag of about 1·5 hr. between curve *A* in Fig. 1 and the curve for the specific activity of the free proline of plasma.) The ratio (0·703) suggests that 70% of the proline of casein came from the free proline of the blood. This figure may not be exact, since the area under the plasma-proline curve cannot be measured accurately; but it may be concluded with certainty that over half the proline residues of casein came from the free proline of the blood. The possibility that the free proline is converted into a derivative, such as a peptide, before being absorbed by the mammary gland may be excluded for reasons similar to those mentioned before (Barry, 1952, 1956).

DISCUSSION

The results which have been described show that the mammary gland takes free proline from the blood to provide at least half of the proline residues of casein. The evidence for the origin of the other non-essential amino acids of casein is as follows. Similar experiments with [^{14}C]glutamine and [^{14}C]glutamic acid, in which areas under specific-activity curves and histograms were compared, gave the result that 100% of the glutamine

and glutamic acid of casein come from the free glutamine and glutamic acid respectively of blood. Allowing for experimental error, we may conclude with certainty that over 70% of the glutamine and glutamic acid of casein come directly from the blood. An experiment with [^{14}C]glucose also showed that less than 20% of the glutamine and glutamic acid of casein were synthesized from glucose within the mammary gland (Barry, 1956). From similar experiments it may be concluded that over 70% of the tyrosine of casein comes from the free tyrosine of blood, and is not derived from plasma proteins, or synthesized from phenylalanine within the gland (Barry, 1952). A further experiment showed that at least 50% of the asparagine residues of casein come from the free asparagine of blood, and that less than 17% are synthesized from glucose within the mammary gland. No significant part of the aspartic acid of casein comes from the asparagine of blood, nor is it synthesized within the gland from glucose; however, its precise origin remains unknown (Sansom & Barry, 1958). Arterio-venous differences in free serine, of up to 48% of the arterial concentrations, were found across the mammary glands of lactating, but not dry, cows (Sheldon-Peters & Barry, 1956). These results suggest that at least part of the serine of casein comes from the free serine of blood. The source in the blood of the cystine, alanine and glycine of casein is unknown, but these amino acids make up only about 6% by weight of the casein molecule.

From these findings it can be calculated that about half, at least, of the residues of non-essential amino acids in casein are derived directly from the blood. It would be interesting to know whether any synthesis of non-essential amino acids occurs in the mammary gland, since it is possible that only a few organs of the body can synthesize some of them, as has been suggested by Eagle, Oyama & Levy (1957). Experiments of the type described in this paper, however, will never exclude the possibility that small amounts of non-essential amino acids are synthesized within the mammary gland.

SUMMARY

1. From an experiment with [^{14}C]proline it is concluded that the mammary gland of the goat takes free proline from the blood to provide at least half of the proline residues of casein.

2. From this and previous work it is calculated that at least half the residues of non-essential amino acids in casein are not synthesized in the mammary gland, but come directly from the blood stream.

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The Reactivity of Free Amino Groups in Native and Denatured Ovalbumin Towards Fluorodinitrobenzene

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Porter (1950) showed that ovalbumin contained no *N*-terminal residue able to react with 1-fluoro-2:4-dinitrobenzene (Sanger, 1945), but subsequent work by Niu & Fraenkel-Conrat (1955) showed that it did contain a *C*-terminal residue (proline). Linderstrøm-Lang (1952) suggested that ovalbumin had one of two possible structures. Anfinsen & Redfield (1956) preferred one of these structures and considered ovalbumin to be a cyclic molecule (probably held by S–S bonds) from which protruded a peptide chain containing the *C*-terminal residue. They suggested that the terminal α-amino group was either masked sterically or chemically bound in a linkage which may be intramolecular or involve the carbohydrate moiety known to be present in ovalbumin (Neuberger, 1938).

The present work describes the effect of increasing amounts of denaturing reagents on the reactivity of ϵ-NH_2 groups of the lysine residues of ovalbumin towards 1-fluoro-2:4-dinitrobenzene. The results indicate that the denaturation of ovalbumin is progressive and suggest that the protein contains three types of lysine residues, differing in their reactivity, or accessibility, towards 1-fluoro-2:4-dinitrobenzene. It is suggested that a structure of the type described by Anfinsen & Redfield (1956) could account for these results.

EXPERIMENTAL

Nessler's quantitative reagent (British Drug Houses Ltd.) and 1-fluoro-2:4-dinitrobenzene (FDNB) (Light and Co., and Kodak Ltd.) were used.

Ovalbumin. This was prepared by the buffered ammonium sulphate method of Cole (1932). The protein was recrystallized six times and finally freed from salt by prolonged dialysis. Analysis of the product (dry and ash-free) gave: total N, 15·72% [cf. 15·76% (Tristram, 1949)]; amide-N, 1·04% [cf. 1·04% (Rees, 1946)].

The crystalline ovalbumin was examined by starch-gel electrophoresis (Smithies, 1955) in borate buffer, pH 8·6 (0·03 M-H_3BO_3; 0·012 M-NaOH). Three distinct bands were clearly distinguishable with only slight differences in electrophoretic mobility. The fractions appeared in approximately the proportions given for A_1, A_2 and A_3 (79, 15 and 6% respectively) by Cann (1949). Since the fractions A_1, A_2 and A_3 are said to contain two, one and no phosphate groups respectively, A_1 should have the greatest mobility at pH 8·6. It was observed that the leading component on starch-gel electrophoresis formed the major fraction of the ovalbumin.

Whole egg white was examined under the same conditions, when it was observed that the albumin fraction became subdivided into three components. These components were eluted together and examined electrophoretically alongside ovalbumin. The eluted fraction separated into three components whose mobilities were identical with those of A_1, A_2 and A_3 respectively of the albumin.

Preparation of 2:4-dinitrophenyl-ovalbumin. The reaction with FDNB is normally carried out in the presence of ethanol (65%, v/v), and considerable denaturation might be expected to occur. A milder reaction may be used in which FDNB is allowed to react at 37° in aq. 8% (w/v) $NaHCO_3$ in the absence of ethanol (Courts, 1954). Under these latter conditions no denaturation takes place.

In the present work four types of reaction with FDNB were carried out (see Table 2): (*a*) in the absence of ethanol without prior denaturation (cf. Courts, 1954); (*b*) in the presence of ethanol without prior denaturation (cf. Sanger, 1945); (*c*) after prior denaturation and removal of the denaturant (ethanol) by aeration at low temperature; (*d*) in the presence of denaturants other than ethanol (guanidine, urea, KI, KSCN). [In all cases the reaction was stopped after a given time by acidification followed by precipitation of the 2:4-dinitrophenyl (DNP)-protein with ethanol.] The product was washed exhaustively with ethanol and ether to remove all traces of FDNB and dried *in vacuo*.

Determination of protein in 2:4-dinitrophenyl derivative by estimation of amide nitrogen. A modified form of the method of Bailey (1937) was used. Five samples of varying weight of DNP-protein (0·5–10·0 mg.) were hydrolysed with 2 N-HCl for 3 hr. under reflux. The hydrolysates were made alkaline to thymolphthalein and made to 100 ml. with glass-distilled water. The NH_3 was removed by micro-Kjeldahl distillation (6 min.) and collected in 2 ml. of 0·01 N-HCl, the final volume being 25 ml. Duplicate 10 ml. portions of the distillate were treated with 2·5 ml. of Nessler's reagent and the extinction was read on an EEL photoelectric colorimeter (Ilford filter no. 303, maximum transmission at 480 mμ). The results were plotted as in Fig. 1*b* and amide N was obtained from the calibration curve obtained by putting $(NH_4)_2SO_4$ through the same procedure (Fig. 1*a*).

Example. The NH_3 from 7·6 mg. of DNP-protein gave an extinction (corrected for blank) of 15·1 (Fig. 1*b*). From the standard curve (Fig. 1*a*) this is equivalent to 28·5 μg. of N. Thus the DNP-protein contains 0·94 g. of amide N/100 g. of dry ash-free DNP-protein. Crystalline ovalbumin contains 1·04% of amide N; hence the DNP-albumin contains 90·5% of ovalbumin.

Analysis of 2:4-dinitrophenyl-ovalbumin. Five samples of each DNP-ovalbumin were hydrolysed with 6 N-HCl (glass-distilled) for 18 hr. After cooling, each hydrolysate was extracted four times with 20 ml. of ether (80 ml. in all). The ether-soluble fractions were pooled and concentrated. Chromatographic analysis on buffered paper (Blackburn & Lowther, 1951) and on Celite columns (Courts, 1954) demonstrated the complete absence of any α-DNP-amino acids. 2:4-Dinitrophenol was detected in all cases and its presence was confirmed by measurement of its ultraviolet-absorption spectrum in dilute acid ($\lambda_{max.}$ 260 mμ) and alkali ($\lambda_{max.}$ 345 mμ).

Chromatographic analysis on silica gel with butan-2-one–ether as mobile phase (Sanger, 1945), and on Celite with the same solvent system (Courts, 1954), showed that all the lysine residues were linked as α-peptides, since only ϵ-DNP-lysine was found in acid hydrolysates of fully substituted DNP-ovalbumin (α-DNP-lysine and ϵ-DNP-lysine have R_F values of 0·11 and 0·20 respectively, with the above systems).

The water-soluble fractions were concentrated by vacuum distillation until all traces of HCl had been removed. Each residue was dissolved in aq. 1% (w/v)

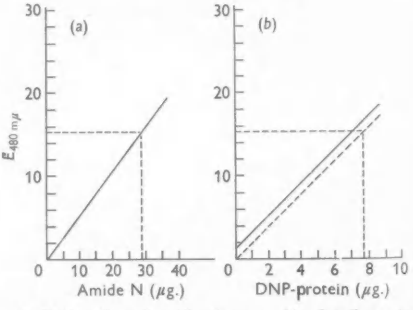

Fig. 1. Estimation of amide nitrogen. For details see text. (*a*) Standard curve. (*b*) DNP-protein: —, uncorrected readings; - - - -, corrected for blank reading.

Table 1. *Reaction of ovalbumin with 1-fluoro-2:4-dinitrobenzene*

The total number of ε-lysine groups/molecule is 20 (Tristram, 1949).

	Prior denaturation				Reaction with FDNB (18 hr.)			
Expt.	Reagent	Concn. (M)	Time (hr.)	Temp.	Ethanol (%, v/v)	Temp.	$NaHCO_3$ (%)	No. of ε-DNP-lysine groups/mol. (45 000)
1	—	—	—	—	—	37°	5	3
2	—	—	—	—	35	20	2	9
3	—	—	—	—	50	20	2	12
4	—	—	—	—	60	20	2	20
5	Urea	8	18	37°	—	37	5	20
6*	Urea	8	18	37	—	37	5	20
7†	Urea	8	18	37	—	37	5	3
8	Guanidine	0·5	4	37	—	37	5	7
9	Guanidine	0·5	0·5	37	50	20	2	11
10	Guanidine	0·75	18	37	—	37	5	20
11	KI	2·5	18	37	—	37	5	11
12	KSCN	2·5	18	37	—	37	5	12

* Preparation no. 1 after reaction with FDNB (three DNP groups) was used in place of crystalline ovalbumin.
† M-Glucose was added as an inhibitor of denaturation. Reaction with FDNB was allowed to proceed for 48 hr.

Table 2. *Progressive denaturation of ovalbumin with ethanol*

Denaturation with ethanol was allowed to proceed for 18 hr. at 37°. After removal of ethanol, the reaction with FDNB was carried out at 37° for 18 hr. in the presence of 5% $NaHCO_3$ (cf. Courts, 1954).

Ethanol (%, v/v)	No. of ε-DNP-lysine groups/mol. (45 000)	Percentage of total groups reacting
0	3	15
10	5	25
20	6	30
30	8	40
40	10	50
50	12	60
55	20	100
60	20	100
70	20	100

$NaHCO_3$ and the absorption measured at 360 mμ. The quantity of ε-DNP-lysine present in each aqueous fraction was calculated from a calibration curve obtained by submitting mixtures of ε-DNP-lysine and ovalbumin to hydrolysis under similar conditions. Hydrolysis with 6N-HCl for 18 hr. led to the degradation of 8% of ε-DNP-lysine and it was therefore assumed that the same loss occurred when ε-DNP-lysine was liberated from peptide linkage.

RESULTS

Ovalbumin (mol.wt. 45 000) contains 20 lysine residues/molecule (Tristram, 1949), all of which can react with FDNB. From the analytical data (Table 1) three main conclusions are apparent: (1) ovalbumin contains no *N*-terminal residue; (2) all the lysine residues have free ε-NH_2 groups, only ε-DNP-lysine being isolated after acid hydrolysis; (3) the extent of the reaction between FDNB and ovalbumin is changed by denaturation (see Expts. 1, 6 and 7.) Complete denaturation (Expt. 5) exposes an additional 17 groups which are not reactive in native ovalbumin (cf. Expt. 1). M-Glucose completely inhibits denaturation (cf. Expt. 7) by urea. [Warner (1954) suggested that ovalbumin was protected from denaturation by sugars, including glucose.]

Ovalbumin contains lysine groups which fall into three classes with respect to their reactivity or availability toward FDNB: (*a*) three readily available residues; (*b*) nine residues reacting only after partial denaturation, and these independently of each other (cf. Expts. 2, 3, 8, 9 and 11); (*c*) eight residues which react simultaneously after denaturation has reached a definite stage (Expts. 4, 5, 6 and 10). Numerous attempts, with no success, have been made to vary conditions so as to obtain a number of ε-DNP-lysine groups intermediate between 12 and 20.

Similar results were obtained in the serial denaturation of ovalbumin with ethanol (Table 2).

DISCUSSION

If the *N*-terminal residue itself is involved in an intramolecular link or is masked, as are the final eight residues of lysine, it would be reasonable to expect that the *N*-terminal group becomes exposed at the same time as these lysine residues. No trace of any α-DNP-amino acid was found, and the terminal amino group is thought to be masked chemically by intramolecular linkage or by

involvement with the carbohydrate moiety (cf. Porter, 1950). Although the acid lability of certain α-DNP-amino acids cannot be ignored (e.g. proline, Porter & Sanger, 1948), no evidence has been obtained for the presence of even traces of α-DNP-amino acids. Bailey (1951) could find only a trace of α-DNP-residue (1 mole/10^6 g. of protein) in four-times-recrystallized ovalbumin.

The availability of lysine residues towards FDNB suggests that the native protein molecule is in the form of a coiled loop, as suggested by Anfinsen & Redfield (1956). The three types of lysine residue with differing reactivity towards FDNB might be accounted for in the following manner: (*a*) three ϵ-NH_2 groups which are in easily accessible sites in the native molecule; (*b*) a further nine groups which cannot react in the native state for steric reasons. As denaturation proceeds the molecule uncoils and these groups become progressively available for substitution. This would indicate that denaturation is a stepwise process, at least with ovalbumin, rather than the all-or-nothing reaction envisaged by Mirsky & Anson (1935); (*c*) as denaturation proceeds a point is reached (50–55% ethanol; 0·5–0·75M-guanidine) when all the remaining ϵ-NH_2 groups interact. This may be an indication of the existence of a cyclic portion of the molecule or that the remaining ϵ-NH_2 groups are in fairly close sequence. It has not been found possible to substitute a number of ϵ-lysine NH_2 groups intermediate between 12 and 20. Thus whereas 50% ethanol exposes 12 groups to reaction, 55% ethanol brings all the 20 groups into a reactive state.

SUMMARY

1. The reaction between ovalbumin and 1-fluoro-2:4-dinitrobenzene has been studied under various conditions.

2. Progressive denaturation before reaction enables three types of ϵ-amino groups with different affinities for 1-fluoro-2:4-dinitrobenzene to be detected.

3. Glucose inhibits denaturation by urea and prevents the substitution of ϵ-amino groups beyond the minimal three groups.

4. Ovalbumin contains no free terminal amino group.

5. All the lysine residues are bound in α-peptide bonds.

6. The significance of these results is discussed.

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Properties and Partial Purification of Kynureninase

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The work to be described originated in a general study of pyridoxal phosphate-linked enzymes. Jakoby & Bonner (1953*a*) obtained kynureninase from *Neurospora crassa* (Em 5256A), studied its properties and claimed to have purified it 70- to 100-fold. This work is being reported as some of their results could not be corroborated. Its special purpose was to study the following problems: (1) To determine the optimum conditions for kynureninase activity. (2) To corroborate the general mechanism of action of pyridoxal phosphate-linked enzymes proposed by Metzler, Ikawa & Snell (1954). To do this it is essential to establish that metal ions are cofactors of these enzymes;

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consequently an attempt has been made to dissociate metal ions from kynureninase. (3) To examine whether kynureninase, like tryptophanase (Gunsalus, Galeener & Stamer, 1955) and some of the amino acid decarboxylases (Steensholt, Flikke & Joner, 1955), is an –SH group enzyme. (4) To attempt to purify the enzyme.

MATERIALS AND METHODS

Kynureninase was obtained from wild-type *N. crassa* (74Q–4b) by the technique of Jakoby & Bonner (1953*a*), with slight modifications.

Growth. The mycelium was produced by allowing *N. crassa* to grow under forced aeration in a 10 l. flask containing 5 l. of minimal medium (Beadle & Tatum, 1945) supplemented with 1·2 g. of DL-tryptophan. Growth was allowed to take place at 30° for 60 hr., the time necessary to reach a wet wt. of 50 g. Mycelium was stored in the deep freeze (−20°) for not more than 8 months.

Extraction of the enzyme. All procedures were carried out at approx. 4° except where otherwise stated. Wet mycelium (50 g.) was cut into small pieces and homogenized with 200 ml. of 0·1M-phosphate buffer, pH 7·8, for 5 min. in an Auto-Mix (M.S.E.). The homogenized suspension was divided equally between two bottles, each containing 300 glass beads (3 mm. diam.), and shaken on a Towers Extra-Rapid Shaker, model E, for 2 hr. The contents of the bottles were strained through cheese-cloth and centrifuged for 35 min. at 17 800 *g* (approximate gravitational field at the bottom of the tube). The volume of the supernatant was made up to 200 ml. with 0·1M-phosphate buffer, pH 7·8. An attempt to extract the enzyme with water was without success. The extract (200 ml.) was submitted to fractionation with ammonium sulphate. Amounts of ammonium sulphate of 36, 24·4, 8·4 and 9·2 g. were added consecutively to give 25, 42, 48 and 54% saturation respectively. The precipitate after each addition was removed by centrifuging at 17 800 *g*. The fractions precipitated at 42–48% and 48–54% were dissolved together in 20 ml. of water (4°) and dialysed for 2 hr. against 4 l. of distilled water. The precipitate which appeared during dialysis was removed by centrifuging and the enzyme solution was stored in a deep-freeze until required. The preparation was active for more than a month.

Purification procedure

An attempt was made to purify the enzyme by the technique of Jakoby & Bonner (1953*a*), with slight modifications. The optimum conditions for the partially purified enzyme were determined before purification was undertaken.

Specific activity. The activity of enzyme preparation in amounts enough to show a linear relationship when plotted against protein concentration was determined in a total volume of 2 ml. The incubation mixture was as follows: 0·5 ml. of 0·05M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·7, 0·203 μmole of pyridoxal phosphate and 4·8 μmoles of DL-kynurenine. Units of specific activity were defined as the μmoles of anthranilic acid formed/min. under standard conditions by 1 mg. of protein.

Preparation and purification of the enzyme. Jakoby & Bonner (1953*a*) extracted kynureninase from the lyophilized mycelium. A freeze-drier was not available when this work was started, therefore the enzyme was extracted by homogenizing the mycelium. When a freeze-drier became available it was noticed that the activity in the extract from the lyophilized mycelium was reduced by 50% compared with the activity in the extract from homogenized mycelium. The detailed procedure for extraction and fractionation of the enzyme has already been given and only the modifications and further steps in purification will be mentioned here.

(i) *Crude enzyme preparation.* Wet mycelium (100 g.) was homogenized with 300 ml. of 0·1M-phosphate buffer, pH 7·8, from which 235 ml. of cell-free extract was obtained containing 2256 mg. of protein with a specific activity of 0·013.

(ii) *Combined ammonium sulphate fractions.* Crude enzyme (235 ml.) preparation was submitted to fractionation with ammonium sulphate and the precipitates obtained between 42–48% and 48–54% of ammonium sulphate saturation were dissolved together in 40 ml. of 0·1M-phosphate buffer. This solution was dialysed against 4 l. of distilled water for 2 hr. and the precipitate which appeared was removed by centrifuging. The supernatant solution contained 228 mg. of protein with a specific activity of 0·05.

(iii) *Protamine sulphate treatment.* To 40 ml. of ammonium sulphate fraction was added 68·4 mg. of protamine sulphate, providing 30 mg. of protamine sulphate for each 100 mg. of protein. The precipitate was removed by centrifuging and the supernatant fluid dialysed for 2 hr. against 4 l. of distilled water. The precipitate, which appears during dialysis, was removed by centrifuging. The protein content of the supernatant was 132 mg. and its specific activity was 0·098.

(iv) *Ethanol treatment.* The enzyme solution (40 ml.) was treated with ethanol by the technique of Jakoby & Bonner (1953*a*). The precipitate obtained by raising the concentration of ethanol to 70% (v/v) was dissolved in 18 ml. of 0·05M-phosphate buffer, pH 7·7. All the precipitated material did not go into solution. The supernatant was dialysed against 4 l. of distilled water for 2 hr. and the precipitate removed by centrifuging. The enzyme preparation (18 ml.) contained 15 mg. of protein. The specific activity determined immediately was 0·032. The activity of the preparation tended to deteriorate rapidly. Thus the ethanol treatment resulted in a fall in the specific activity.

Method of measuring the enzyme activity. Tubes containing 2·4 μmoles of DL-kynurenine, 0·5 ml. of 0·05M-tris buffer, pH 7·7 (or 0·5 ml. of the buffer under test), and 0·25 ml. of enzyme in a final volume of 2 ml. were incubated at 37° for 1 hr. At the end of the incubation period the reaction was stopped by the addition of 4 ml. of ethanol and the precipitate removed by centrifuging. Each determination required one blank tube containing all the constituents in the reaction tube, with deproteinizing agent added at zero time, which was subsequently treated in the same manner as the reaction tube. The optical density of the supernatant solutions was measured in a 1 cm. cell at 310 mμ in a Unicam spectrophotometer model SP. 500. The amount of anthranilic acid formed was read from a calibration curve.

Protein estimation. The protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). The

calibration curve was prepared with crystalline bovine plasma albumin (The Armour Laboratories, Chicago, Ill., U.S.A.).

Dialysis. The technique of dialysis was that of Spiegelman, Reiner & Morgan (1947). Enzyme preparations were dialysed in cellophan bags rotated at an angle of 45° in the dialysis liquid; an air bubble was left in the bag to increase the circulation of its contents. The dialysis was done in the cold room (4–5°).

Buffers. The following buffers were used: phosphate (Sørensen, 1909); citrate–phosphate (McIlvaine, 1921); borate (Clark, 1922); universal-buffer mixture (Johnson & Lindsey, 1939); 2-amino-2-hydroxymethylpropane-1:3-diol (tris) and veronal (Gomori, 1955).

Chemicals. DL-Kynurenine and analogues of vitamin B_6 (Roche Products Ltd.). *p*-Chloromercuribenzoic acid (Hopkin and Williams Ltd.). L-Cysteine, reduced glutathione and tris (L. Light and Co. Ltd., Colnbrook, Bucks.). Biotin, diethylbarbituric acid and sodium barbitone (British Drug Houses Ltd.). Anthranilic acid was recrystallized several times from hot water until the sample had a constant m.p. at 145°. Other chemicals used were A.R. grade.

Measurement of pH. The pH of buffers was determined with a Marconi type T.F. 511D pH meter, buffer tablets (Marconi Instruments Ltd.) being used as standard.

RESULTS

Time. The effect of variation of time (15–240 min.) on the enzyme reaction is shown in Fig. 1.

Effect of pH *and buffers.* The curves relating activity to pH and buffers (Fig. 2) show an optimum pH at 7·7 and also reveal that at pH 7·3 the activity in borate and universal-buffer mixture is 51·8 % of that in phosphate and citrate–phosphate buffers at the same pH. An experiment with a view to observing the comparative effect of 0·0125 M-tris, 0·0125 M-veronal and 0·025 M-phosphate buffer on the enzyme activity showed that the optimum pH of the enzyme activity in all the buffers was at 7·7; the activity in tris and veronal buffers was 41·5 % of that in phosphate buffer, comparison being made at pH 7·7. The increased activity in phosphate buffer may be due to the activation of the enzyme by some component of the phosphate buffer. Neither sodium chloride nor potassium chloride affected enzymic activity. Sodium phosphate, pH 7·8, at concentrations above 0·1 mM activated the enzyme, the maximum activation being 78·4 %, which was observed at 10 mM-phosphate in tris buffer, pH 7·8.

Substrate concentration. Fig. 3 shows the effect of varying the substrate concentration on the enzymic activity in the presence of 0·0406 μmole of pyridoxal phosphate/ml.; it shows that 2·4 μmoles of DL-kynurenine/ml. are required for optimum activity and DL-kynurenine is not inhibitory even at a concentration of 4·8 mM.

Coenzyme. The saturation curve (Fig. 4) shows that 0·0406 μmole of pyridoxal phosphate/ml. is needed for optimum activity and that it is not inhibitory at 0·2 mM concentration. The dissociation constant is 0·35 μM. Table 1 shows that the activating effects of pyridoxal phosphate and phosphate were not additive.

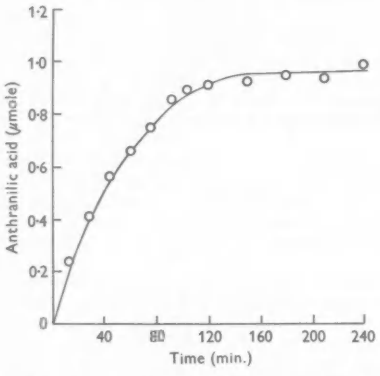

Fig. 1. Amounts of anthranilic acid formed with the time of enzyme reaction. Production of anthranilic acid is linear with time up to 1 hr.

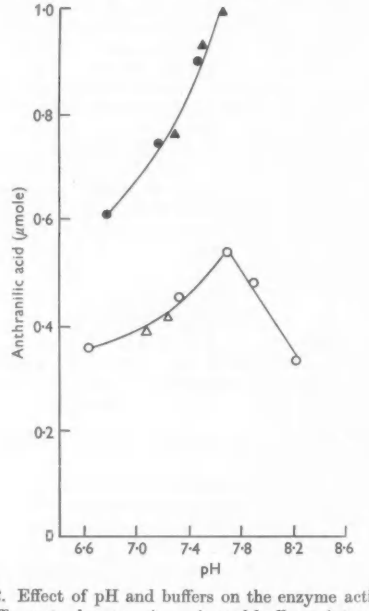

Fig. 2. Effect of pH and buffers on the enzyme activity. Buffers: ○, borate; △, universal-buffer mixture; ●, citrate–phosphate; ▲, phosphate.

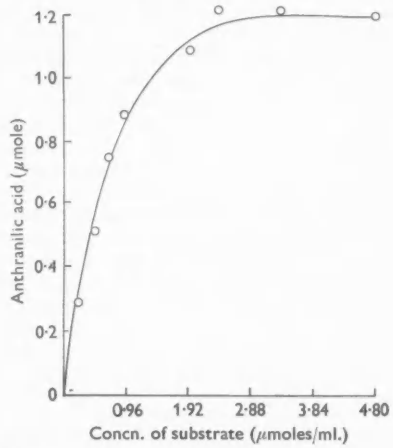

Fig. 3. Effect of substrate concentration on the enzyme activity. Activity was determined in the presence of pyridoxal phosphate (0·0406 μmole/ml.). DL-Kynurenine (2·4 μmoles/ml.) is necessary for optimum activity.

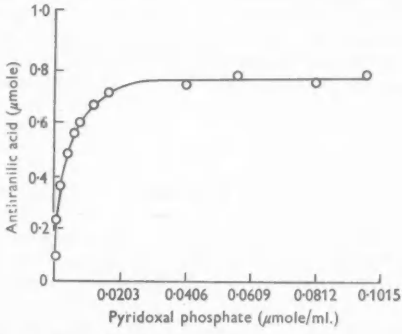

Fig. 4. Pyridoxal phosphate-saturation curve. The assay was conducted under standard conditions except for the variation of pyridoxal phosphate. Pyridoxal phosphate (0·0406 μmole/ml.) is necessary for optimum activity.

Table 1. *Effect of pyridoxal phosphate and sodium phosphate* (pH 7·8) *on the enzyme when tested separately and combined together*

Activity was determined in tris buffer, pH 7·8.

Expt. no.	Sodium phosphate (mM)	Pyridoxal phosphate (μmole/ml.)	Activity (% of original)
1	0	0	100
2	10	0	166·2
3	0	0·0609	203·2
4	10	0·0609	203·2

Metal ions. Tryptophanase and glutamic acid decarboxylase, which are pyridoxal phosphate-linked enzymes, are activated by some univalent cations (Happold & Struyvenberg, 1954; Happold, 1955). The chlorides of sodium, potassium, ammonium and rubidium at concentrations from mM to 0·1 M were without effect on kynureninase activity. The effect of varying concentrations of some bivalent metal ions both in the presence and absence of 0·203 μmole of pyridoxal phosphate was also determined. Cobalt chloride, magnesium sulphate, ferrous sulphate and zinc sulphate at concentrations above 0·01 mM inhibited the enzyme activity both in the presence and absence of pyridoxal phosphate. Calcium chloride activated both in the presence and absence of pyridoxal phosphate at concentrations above 0·01 mM, and the optimum activity was observed at mM; the activation varied from 5 to 56% with different batches of the enzyme preparation. Manganese sulphate, in the presence of pyridoxal phosphate, activated kynureninase at concentrations from 5 μM to 0·5 mM; the optimum activation was 33·6%, which was obtained at 10 μM concentration. At concentrations greater than 0·5 mM this salt inhibited and in the absence of pyridoxal phosphate it was inhibitory at concentrations greater than 10 μM.

A large number of techniques have been used for dissociating metal ions from enzymes (Smith, 1948; Dagley & Sykes, 1956). Attempts to dissociate the metal ions from kynureninase by dialysing the enzyme against (*a*) 0·02 M-ethylenediaminetetra-acetic acid (EDTA) adjusted to either pH 7·2 or 4·5 for 24 hr., (*b*) 0·1 M-phosphate–citrate buffer, pH 7·6, for 12 hr., (*c*) distilled water for 48 hr. were all only partially successful. The enzyme dialysed against EDTA (*a*) or phosphate–citrate (*b*) was activated by Mn^{2+} ions to the same extent as the undialysed enzyme. The enzyme dialysed against distilled water and the undialysed preparation were activated by Mn^{2+} ions to the extent of 27·2 and 15·5% respectively.

Effect of analogues of vitamin B_6. Before the addition of the substrate, the enzyme, buffer and analogue of vitamin B_6 (25 μg./ml.) were incubated for 45 min. at 37°. Pyridoxal did not affect the enzyme activity; pyridoxamine inhibited by 14%; and pyridoxamine phosphate and pyridoxal phosphate activated the enzyme by 104 and 150% respectively. The results of varying the concentration of pyridoxamine phosphate are shown in Table 2.

Pyridoxamine phosphate transaminase catalyses the conversion of pyridoxamine phosphate into pyridoxal phosphate (Beechey & Happold, 1955). The activation of kynureninase by pyridoxamine phosphate may be due to the presence of pyridox-

amine phosphate transaminase. However, the presence of such an enzyme could not be demonstrated in the kynureninase preparation by the technique of Beechey & Happold (1955).

Evidence for the presence of functional sulphydryl groups. Steensholt *et al.* (1955), Gunsalus *et al.* (1955) and Sher & Mallette (1954) have shown that glutamic acid decarboxylase, tryptophanase and lysine decarboxylase, all of which are pyridoxal phosphate-linked enzymes, require the integrity of –SH groups in their structure for their activity. *p*-Chloromercuribenzoate specifically inhibits enzymes, dependent for activity on the presence of –SH groups (Hellerman, Chinard & Deitz, 1943). Sher & Mallette (1954), for example, inactivated lysine decarboxylase by *p*-chloromercuribenzoate and were able to maintain the activity at 80 % of the original activity by incubating the enzyme with 100-fold excess of cysteine for 20 min. before the addition of *p*-chloromercuribenzoate. The other enzymes inactivated by *p*-chloromercuribenzoic acid have been shown to be reactivated by reduced glutathione (Barron, 1943). Iodoacetic acid is also known to inactivate enzymes depending for activity upon reduced –SH groups (Hopkins, Morgan & Lutwak-Mann, 1938). The activity of this compound was tested as follows: 50 ml. of 0·5 M-iodoacetic acid was adjusted to pH 7·7 with sodium hydroxide and the volume made up to 100 ml. The enzyme preparation was then incubated at room temperature with iodoacetate (12·5 mM), pyridoxal phosphate (0·203 μmole) and buffer for 2 hr. before determining the activity. Iodoacetate inhibited the enzymic activity by 80 %.

Reversibility of p*-chloromercuribenzoic acid inactivation by reduced glutathione and* L*-cysteine.* The enzyme preparation was incubated at room temperature with varying concentrations of *p*-chloromercuribenzoic acid, pyridoxal phosphate (0·203 μmole) and buffer for 2 hr. before determining the enzymic activity. *p*-Chloromercuribenzoic acid inhibited activity at concentrations above 10 μM and there was 93 % inhibition at 0·1 mM.

Reduced glutathione was incubated with the enzyme, pyridoxal phosphate (0·0813 μmole) and buffer for 20 min. before the addition of *p*-chloromercuribenzoic acid. The concentrations of glutathione and *p*-chloromercuribenzoic acid and their effect on the enzymic activity are presented in Table 3, which shows that reduced glutathione counteracts the inhibitory effect of *p*-chloromercuribenzoic acid and that the activity is maintained at 99·4 % of its initial value. *p*-Chloromercuribenzoic acid alone inhibits the enzymic activity by 89·7 % in this experiment.

A solution of L-cysteine hydrochloride was neutralized to pH 7·0 with sodium hydroxide and diluted to the required concentration. A concentration of L-cysteine at 10 mM inhibited the kynureninase activity by 47 % (Jakoby & Bonner, 1953*b*). The inhibition was thought to be due to the formation of a cyclic compound between L-cysteine and pyridoxal phosphate. It was therefore considered desirable to add pyridoxal phosphate in excess to minimize the inhibitory effect of L-cysteine. L-Cysteine was incubated with the enzyme, buffer and pyridoxal phosphate (0·325 μmole) for 20 min. before the addition of chloromercuribenzoic acid. The concentration of L-cysteine and chloromercuribenzoic acid and their effect on the enzymic activity are given in Table 4. L-Cysteine did not inhibit the enzymic activity, whereas the 90 % inhibition caused by chloromercuribenzoic acid was almost completely reversed by incubation of the enzyme with cysteine for 20 min. before the addition of *p*-chloromercuribenzoic acid.

Table 2. *Effect of varying the concentration of pyridoxamine phosphate on the enzymic activity*

Expt. no.	Pyridoxamine phosphate (μmole/ml.)	Activity (% of original)
1	0	100
2	0·0202	197·3
3	0·0404	236·8
4	0·0607	250·0
5	0·1012	276·3

Table 3. *Effect of reduced glutathione on the inhibitory effect of chloromercuribenzoic acid*

Pyridoxal phosphate (0·0813 μmole) was present.

Expt. no.	Addition	Molarity (mM)	Activity (% of original)
1	None	—	100
2	Reduced glutathione	20	97·3
3	*p*-Chloromercuribenzoic acid	0·1	10·3
4	Reduced glutathione + *p*-chloromercuribenzoic acid	20 0·1	99·4

Table 4. *Effect of* L*-cysteine on the inhibitory effect of chloromercuribenzoic acid*

Pyridoxal phosphate (0·325 μmole) was present.

Expt. no.	Addition	Molarity (mM)	Activity (% of original)
1	—	0	100
2	L-Cysteine	1·5	102·5
3	*p*-Chloromercuribenzoic acid	0·1	10·2
4	L-Cysteine + *p*-chloromercuribenzoic acid	1·5 0·1	97·4

DISCUSSION

It has been shown that the increased enzymic activity in phosphate as compared with that in borate, tris, universal and veronal buffer is due to the activating effect of the phosphate radical. Jakoby & Bonner (1953*a*) observed the same degree of kynureninase activity in phosphate, tris and borate buffers. This difference has been satisfactorily explained since it was observed that phosphate did not activate kynureninase in the presence of added pyridoxal phosphate. Jakoby & Bonner (1953*a*) determined the effect of different buffers on the enzymic activity in the presence of added pyridoxal phosphate.

Jakoby & Bonner (1953*b*) stated that the inhibition by higher concentrations of the substrate, in the presence of optimum amounts of added pyridoxal phosphate, was due to the formation of a 'kynurenine–pyridoxal phosphate complex'. The formation of this complex lowered the effective pyridoxal phosphate concentration and the complex itself was thought to act as an analogue type of inhibitor. Although D-kynurenine did not act as substrate, it was as inhibitory as its enantiomorph; L-kynurenine inhibited at concentrations above 0·4 mM. In the present study DL-kynurenine in the presence of an optimum amount of pyridoxal phosphate was found not to inhibit the enzymic activity even at 4·8 mM. No satisfactory explanation could be given for the difference between the present results and those of Jakoby & Bonner (1953*b*). However, it may be mentioned that DL-kynurenine has been used as substrate here, whereas Jakoby & Bonner (1953*b*) used L-kynurenine sulphate.

Jakoby & Bonner (1953*a*) found that pyridoxal phosphate inhibited kynureninase activity at concentrations above 4 μM and that the dissociation constant derived by determining the coenzyme concentration at half-maximum velocity was 0·6 μM. The present work shows that pyridoxal phosphate even at 0·2 mM is not inhibitory and the dissociation constant of the coenzyme has a value of 0·35 μM.

Jakoby & Bonner (1953*a*) claimed that Mg^{2+} ions at a concentration of 2 mM activated kynureninase at all stages of purification, the activation being of the order of 15–45%, depending upon the preparation, and Ca^{2+} ions also activated at some stages of purification; the response was highly variable. The present work shows that Mn^{2+} and Ca^{2+} ions activate the enzyme. It seems probable that kynureninase obtained from different strains of wild-type *N. crassa* is not activated by the same metal ions.

Of the various analogues of vitamin B_6 tested, only pyridoxal phosphate and pyridoxamine phosphate activated the enzyme. Jakoby & Bonner (1953*a*) reported that pyridoxamine phosphate did not activate kynureninase. The possibility that pyridoxamine phosphate might also act as a coenzyme of kynureninase should also be borne in mind—especially in view of the fact that kynurenine contains a carboxyl group. Meister, Sobar & Peterson (1954) reported that purified pig-heart glutamic–aspartic apotransaminase is activated by pyridoxal phosphate and pyridoxamine phosphate.

Evidence has been presented for the first time that kynureninase possesses functional –SH groups. Some of the other pyridoxal phosphate-linked enzymes, such as glutamic acid decarboxylase, leucine decarboxylase and tryptophanase, possess functional –SH groups.

SUMMARY

1. Production of anthranilic acid is linear with time up to 1 hr.

2. The pH optimum of kynureninase was 7·7 and the optimum activity was observed in phosphate buffer, which was shown to be due to the activating effect of phosphate radical. Phosphate did not activate the enzyme in the presence of added pyridoxal phosphate.

3. DL-Kynurenine even at a concentration of 4·8 mM in the presence of an optimum amount of pyridoxal phosphate did not inhibit the enzymic activity.

4. Pyridoxal phosphate at a concentration of 0·2 mM did not inhibit the enzymic activity.

5. Mn^{2+} ions activated kynureninase only in the presence of added pyridoxal phosphate, whereas Ca^{2+} ions activated both in the presence and absence of added pyridoxal phosphate.

6. Of the various analogues of vitamin B_6 tested, only pyridoxal phosphate and pyridoxamine phosphate activated the enzyme. Pyridoxamine was slightly inhibitory.

7. Evidence has been presented for the presence of functional –SH groups in kynureninase.

8. Kynureninase was purified eightfold.

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The Metabolism of DL-1-Deoxy-1-Fluoroglycerol

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The study of fluorine-substituted metabolites may give information about the pathways of normal metabolism or lead to the discovery of compounds suitable for therapeutic or pesticidal use. The compound 3-fluoro-DL-propane-1:2-diol (DL-1-deoxy-1-fluoroglycerol) was first described by Gryskiewicz-Trochimowski (1947), and its physical properties were given by Taylor & Kent (1956). Dr R. M. C. Dawson (personal communication) observed that the compound was toxic to mice and induced citrate accumulation, particularly in heart and kidney, and to a lesser extent in liver. Since the present work was started, Pattison & Norman (1957) have reported that DL-1-deoxy-1-fluoroglycerol is toxic to the mouse, and have suggested that it is oxidized *in vivo* to fluoroacetate or fluoroacetaldehyde, on the grounds of a comparison of its toxicity with that of various possible metabolites of DL-1-deoxy-1-fluoroglycerol or of their derivatives.

MATERIALS AND METHODS

DL-1-Deoxy-1-fluoroglycerol (FG) and epifluorohydrin were synthesized by Drs Taylor and Kent and generously presented to us by them. [Dr Kent found (for FG): C, 38·0%; H, 7·6%. Calc. C, 38·3; H, 7·45%.] Sodium fluoroacetate was purchased from the Monsanto Chemical Co. The disodium salt of adenosine triphosphate (ATP) was used, purchased from the Pabst Laboratories, Milwaukee, Wisc., U.S.A. Citrate was determined by the method of Taylor (1953), except that the step of drying the light petroleum was omitted. Phosphate was determined by the method of Rockstein & Herron (1951). Acid-labile phosphate was determined following 10 min. hydrolysis in N-HCl at 100°. Glycolysis was measured according to LePage (1948). Kidney particles were prepared and assayed, with fumarate as substrate, as described by Peters & Wakelin (1953, 1957); brain particles were used as described by these authors. Metabolic products were examined for organic acids, by two-dimensional paper and by column chromatography.

Paper chromatography. The two-dimensional hydroxamate system of Bergmann & Segal (1956) was used; the first solvent was absolute ethanol–pyridine–water–ammonia (60:20:6:14, by vol.) and the second was absolute ethanol–dioxan–water–acetic acid (60:20:19:2, by vol.). At the suggestion of Dr D. B. Lindsay, the free acid for chromatography was obtained by evaporating a solution of the sodium salt to dryness, taking up in 75 μl. of 30% H_3PO_4, adding to a micro-column (0·6 cm. × 1·5 cm.) of anhydrous Na_2SO_4 topped with Celite 545, and washing through with ether.

Column chromatography. The material to be chromatographed was made alkaline and evaporated to dryness on the steam bath, then taken up in 0·5 ml. of 0·5N-H_2SO_4, and chromatographed by the silica-gel-column procedure of Bulen, Varner & Burrell (1952), eluting only with butanol–chloroform (5:95, v/v). Successive 2 ml. fractions were

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collected and neutralized. In fractionating a heart–liver perfusate, the neutral emulsions were evaporated to dryness on the steam bath, each was dissolved in 1·25 ml. of 0·25M-phosphate buffer (pH 7·2), and 1 ml. was assayed, by the standard kidney-particle preparations, for compounds which could be converted into fluorocitrate.

Perfusions. The coronary-perfused heart preparation of Bleehen & Fisher (1954) was used upon female rats anaesthetized with Nembutal (48 mg./kg.) and injected with heparin (2000 units). The heart–liver preparation was essentially a combination of the Bleehen & Fisher perfused heart with liver perfused according to Trowell (1942), with adaptations based on Brauer, Pessotti & Pizzolati (1951) and Miller, Bly, Watson & Bale (1951), the organs being arranged in parallel. Both organs were obtained from a single heparinized rat under Nembutal anaesthesis, the heart being stored in iced Ringer's solution until the liver was in the apparatus. Fig. 1 shows the apparatus. The ischaemic interval was not more than 10 min. Penicillin G sodium (Glaxo Laboratories Ltd.) (65 000 units) was added to the 55–70 ml. of perfusion fluid, which was that used by Bleehan & Fisher.

Functional hepatectomy. 'Eviscerations' (removal of the alimentary canal and those tissues between the rectum and the lower oesophagus which are connected to it) were carried out under urethane anaesthesia (1·5 g./kg.). When total functional hepatectomy was desired, the following were tied: rectum; oesophagus; coeliac artery; inferior and superior mesenteric arteries; and finally the portal vein. The rat was then eviscerated and sewn up. Subcutaneous injections of penicillin G sodium (33 000 units) were made immediately after the operation (Markowitz, Rappaport & Scott, 1949) and of glucose (200 mg.) at 2–3 hr. intervals. When the hepatic arterial supply was to be retained, the same procedure was used except that, instead of the coeliac artery, its non-hepatic tributaries (lineal, gastroduodenal and left gastric) were tied; in this case penicillin, but no glucose, was injected. Tracheotomy was performed when necessary. FG (dissolved in 0·9% NaCl) was injected into the femoral vein.

Rats. Rats were either those bred at this Institute from a Wistar strain obtained originally from the National Institute for Medical Research, Mill Hill, London, N.W. 7; or they were purchased from A. Tuck and Sons, Rayleigh, Essex. The former rats were used for the perfused hearts and livers. About equal numbers of each were used for the hepatectomy experiments.

RESULTS

Since the amount of the substance available was small, experiments had to be designed with a view to economy of DL-1-deoxy-1-fluoroglycerol.

Effect in vivo. DL-1-Deoxy-1-fluoroglycerol (FG) was toxic when injected into the rat, mouse and guinea pig, as shown in Table 1. The values obtained represent the order of toxicity. As the amounts of fluoroglycerol were so limited, no attempts was made to get an exact LD_{50}. Death was associated with considerable citrate accumulations (Table 2). The symptoms of poisoning in rats injected with an approximate LD_{50} are as follows: no signs for about 1 hr., then a brief extreme hyper-

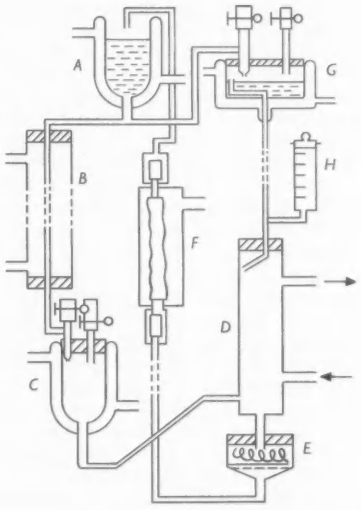

Fig. 1. Heart–liver perfusion apparatus. The rubber tubes with screw clamps inserted in the tubing between *A* and *B*, *B* and *C*, *C* and *D*, *A* and *G*, *H* and *D* are not shown. Chambers *A*, *B*, *C* and *G* are jacketed with water pumped by a centrifugal pump from a thermostat bath at 38°. Perfusion fluid in the reservoir (*A*) may flow through *B* into the cannula on which is tied the heart, in chamber *C*. After passing through the heart, it flows through the bottom of *D* into *E* (a Büchner funnel packed with glass wool and with a Whatman no. 351 paper at the bottom), and then through the pump *F*. The pump is provided with two dental valves, at entry and exit, and a gum-rubber tube in the centre, which responds to the alternating pressure created in the outer chamber of *F* by connexion to a Dale–Schuster pump (not shown). The fluid then returns to *A*, which is treated with Silicone M.S. Antifoam A (Hopkin and Williams Ltd.). Other fluid from *A* passes into the cannula in *G*, on which the liver is tied, perfusion being through the thoracic vena cava and out of the portal vein (the abdominal vena cava is tied). The liver lies in *G* supported by perfusion fluid, which overflows down the outflow tube. *H* is used to measure the flow rate through the liver, by clamping the *H*–*D* clamp and removing the stopper of *H*. Normally, fluid flows straight from *G* into *D*, the gassing tower, flowing down the wall and into *E*. Tower *D* is gassed with O_2+CO_2 (95:5, v/v) in the direction shown by the arrows. The heart flow-rate is measured by introducing an air bubble with polythene cannula tubing, and measuring its speed through *B*, by using calibrated marks on *B*. Important dimensions: liquid level in *A* is 76 cm. above the heart in *C* and 5·5 cm. above the liver in *G*. Chamber sizes (internal diameter × internal depth in cm.): *A* and *C*, 2·6 × 5·0; *G*, 4·3 × 3·0. In later experiments a filter of stainless-steel mesh (Baxter N 14 Filter-drip Assembly; Baxter Lab. Inc., Morton Grove, Illinois) was inserted between *A* and *B*.

excitability ending in clonic convulsions which often terminate in a tonic convulsion. During the next 3–4 hr., several similar convulsions occur, often within a few minutes of each other, the tonic phase becoming more dominant and severe. In between convulsions there is lassitude, although the animals are lively on handling, and fur staring; rectal temperatures are normal at 33–35°, as taken under the conditions in this Laboratory; hearts are normal at 500 beats/min. After this 3–4 hr. phase there are seldom any more convulsions until death, but rectal temperatures and heart rates decline slowly until at 28 hr. they average 25° and 150 beats/min. respectively. The heart beat is then very faint and irregular, the animals sprawl, with hind legs particularly splayed out. They are weak and completely lethargic, even on handling when they are apt to appear resentful. Often a little red secretion is observed at eyes and nostrils. They do not eat or drink. By 44 hr. those that are going to survive are recovered with heart rates at about 450 beats/min., temperatures 34°; they eat, drink and are lively. Those that will die eventually may make a partial recovery (e.g. to temperature 31°, heart 150 beats/min.), but they do not eat and become colder once more; they may convulse on handling and eventually die at about 44 hr. with temperatures of 20–25°, about half of them with a mild tonic convulsion immediately before death.

Citrate analyses upon the tissues are given in Table 2. The citrate content of all four tissues studied was increased, the heart being most affected, but there was very considerable individual variation in response. At the presumed LD_{50} of 10 mg./kg. the survivors had less citrate in all tissues than had those which succumbed, and this difference was especially striking with respect to the heart. The survival time after injection decreased sharply with increasing dosage. When citrate determinations were made on the tissues removed 3 hr. or more after death, the values for poisoned animals were radically reduced. Thus the citrate level in freshly removed hearts of animals that had succumbed to 10 mg. of FG/kg. averaged 1173 μg./g.; but, in two similar animals in which tissues were removed 3 and 5 hr. after death, values of 97 and 136 μg./g. respectively were obtained. A somewhat similar phenomenon was observed by Buffa & Peters (1949).

Paper chromatography. The sample of FG was examined chromatographically for possible contamination with fluoroacetate. The conditions were

Table 1. *Toxicity of* DL-1-*deoxy*-1-*fluoroglycerol to the mouse, guinea pig and rat*

Injections were intraperitoneal.

Animal	Sex	Average wt. and range (g.)	Dose (mg./kg.)	No. injected	No. died	Estimated LD_{50}
Mouse	M.	36 (26–42)	50	2	2	20–40
			45	4	4	
			40	4	4	
			20	4	0	
			10	4	0	
Guinea pig	F.	596 (450–666)	20	4	4	<20
Rat (Tuck)	M.	174 (139–228)	100	2	2	25
			50	3	3	
			25	3	2	
			20	2	0	
			10	4	0	
Rat (Babraham)	M.	141 (80–206)	100	2	2	10
			20	5	5	
			10	8	5	

Table 2. *Effect of intraperitoneal injection of* DL-1-*deoxy*-1-*fluoroglycerol in male rats*

Rats were those bred at Babraham. Citrate was determined on extracts treated with trichloroacetic acid, 5% final concentration. Figures are means ± S.D.

Dose (mg./kg.)	No. of animals	Time to death (hr.)	Citrate (μg./g. wet wt.)			
			Heart	Liver	Kidney	Brain
0	3	—	54±9	18±1	37±1	43±7
10 (survivors)*	2	—	41, 93	31, 33	79, 93	45, 46
10 (succumbed)	2	23, 42	1688, 760	70, 57	217, 147	136, 108
20	4	25±2	2537±658	164±80	926±296	388±131
100	1	8	1245	224	1140	160

* Killed 44 hr. after injection.

such that 1% of fluoroacetate would have been detected. None was found.

At high levels of FG (e.g. 250 μg.) a purple spot with R_F 0·81 in the alkaline and 0·78 in the acid solvent was noted. This was certainly not due to fluoroacetate, which had R_F values of 0·057 and 0·63 in alkaline and acid solvents respectively. No attempt was made to identify the purple spot, which may have been some product of FG.

Taken with the observation below of the inactivity of the FG against kidney particles, it is certain that no significant amount of fluoroacetate can be present in the sample, and that the effects of FG are not due to contamination of the sample with fluoroacetate.

Results with tissue homogenates and slices. The symptoms of poisoning and the great increases in citrate levels in the tissues suggested that the critical biochemical lesion occurred with aconitase, as is the case for fluoroacetate (Lotspeich, Peters & Wilson, 1952; Morrison & Peters, 1954). If this were so, the FG was probably being converted into fluorocitrate, and fluoroacetate or fluoroacetyl coenzyme A were possible intermediates.

All attempts to duplicate the citrate accumulation with simple enzymic preparations *in vitro* were unsuccessful. At 2 mM, FG had no effect on oxidation of fumarate or pyruvate plus oxaloacetate by kidney or brain particles, nor was any citrate accumulation found in the preparations. Such particles are very sensitive to fluoroacetate at 0·1 mM. Since it was possible that a preliminary conversion into (say) fluoroacetate took place *in vivo* in some other tissue, mixed preparations of kidney with liver, heart or voluntary muscle were tried, both homogenates and slices. Also di- and tri-phosphopyridine nucleotide, nicotinamide and fluoride along with simple or mixed slice and homogenate preparations were studied. No citrate accumulations were found.

At 7 mM, FG does not affect the extra oxygen uptake obtained by kidney slices on the addition of glycerol, citrate or pyruvate. No oxidation of FG by liver slices could be observed manometrically. Much time was spent on reaching this negative conclusion and many variations of media were used, i.e. Krebs–Henseleit bicarbonate and phosphate, and three improved media suggested by Krebs and colleagues (Krebs & Henseleit, 1933; Krebs, 1950). Extracts of liver, kidney or heart slices incubated for up to 3 hr. with FG did not contain any material that gave citrate accumulation after incubation with kidney particles. It might be thought that any fluoroacetate could be detected by paper chromatography, but in this Laboratory it has proved very difficult in the products of biochemical incubation mixtures to distinguish fluoroacetate and especially to separate this from acetate. Hence the failure to induce citrate accumulation in kidney preparations is more reliable evidence for the absence of fluoroacetate.

The possibility that FG might influence glycerol metabolism or glycolysis was investigated. With FG at 7 mM (21 μmoles/flask) guinea-pig-brain glycolysis was inhibited slightly as follows: (*a*) no added substrate: CO_2 evolution inhibited 22%; lactate formation 13%; phosphorus incorporation increased by 0·8 μmole: (*b*) glucose as substrate: CO_2 evolution inhibited 7%; lactate formation 5%; phosphorus incorporation increased 0·7 μmole. (Results are means of three experiments.) This suggested the possibility of some phosphorylation of FG, perhaps with small subsequent interference with glycolysis. However, with purified rat-liver glycerokinase (Bublitz & Kennedy, 1954) FG was not phosphorylated nor did it interfere with phosphorylation of glycerol (4 mM-FG with 10 mM-glycerol).

Perfused heart. The failure of the sliced or homogenized tissues to show the citrate accumulation with FG that they did *in vivo* suggested that an intact perfused organ might repay study. The heart is easy to perfuse, and showed considerable increases of citrate with FG *in vivo*. Also it survives for many hours, and one might observe effects in it (due to an enzyme system of low concentration or of low affinity for FG) that sliced tissue would not demonstrate. The coronary-perfused heart was examined: heart beat and perfusion rate were noted for 5 hr. (or less if the heart stopped sooner), then it was removed and ground with trichloroacetic acid, and citrate determined on the extract. Table 3 shows that although 1·2 mM-fluoroacetate caused marked citrate accumulation, 4 mM-FG had no effect at all.

Table 3. *Effect of fluoroacetate and* DL-1-*deoxy*-1-*fluoroglycerol on the coronary-perfused rat heart*

The citrate content of the tissue was determined after 5 hr., or when the heart had stopped (if before 5 hr.). The figures represent means ± S.D.

Addition to perfusion fluid	No. of experiments	Citrate (μg./g.)	After 2 hr. Heart rate (beats/min.)	After 2 hr. Flow rate (ml./min.)
Nil	4	42 ± 15	181 ± 37	2·3 ± 0·9
Sodium fluoroacetate (1·2 mM)	3	179 ± 72	74 ± 58	1·3 ± 0·8
FG (4·0 mM)	3	50 ± 13	213 ± 37	3·3 ± 0·9

Since the perfused beating heart is a more 'physiological' preparation, its failure to act when isolated as it did in the whole animal could most readily be explained by the assumption that, *in vivo*, FG must be altered or 'activated' by some other tissue than heart, before it was capable of accumulating citrate in the heart.

Functional hepatectomy. As attempts to obtain comparable results *in vivo* and *in vitro* had failed, it was decided to attempt the opposite approach: rather than obtain an enzymic preparation *in vitro* complex enough to metabolize FG, an attempt would be made to eliminate various organs in the intact animal in order to establish their importance by observing the effect of FG injected subsequently. The liver was selected as the first organ to study.

There are two problems in connexion with hepatectomy in the rat, owing to the facts that (*a*) the venous return from the alimentary canal goes through the liver, by the portal vein, (*b*) part of the vena cava is in intimate contact with the liver. In order to remove the liver, a preliminary operation would be required in which the vena cava and portal vein are constricted in order to develop collateral circulation. Six weeks must elapse before the hepatectomy can be performed (Cheng, 1951). Even so, the animals survive only 6 hr.

A method was therefore sought to accomplish a rapid functional hepatectomy with reasonable survival. The details of the procedure eventually evolved are given under Materials and Methods. The warnings of Markowitz (1949*a*) were, however, borne in mind: tying off the portal vein and hepatic artery does not completely eliminate the liver from the circulation (presumably owing to diffusion into and out of the vena cava), and dogs treated in this way, even though provided with an Eck fistula, survive only 10–12 hr. and do not show the typical symptoms of hepatectomy.

If the portal vein is tied the animal dies rapidly (e.g. 1·5 hr.) owing to loss of blood from the general circulation into the vessels of the portal system. Therefore to improve survival the blood supply to the alimentary canal must be stopped; and since decomposition of the canal presumably begins when the circulation stops it was considered best to remove the canal and its adherent organs altogether (this is termed 'evisceration' in this paper, although the term would usually imply removal of the liver as well). As Table 4 (*a* and *b*) shows, animals so treated survive for over 12 hr., a period longer than that required for intact animals to be killed by 85 mg. of FG/kg. Yet, as Table 4 shows, this very high dose of FG had no effect on the survival time or the citrate levels of kidney or heart of these eviscerated and functionally hepatectomized animals. This proved that either the liver or the alimentary canal is essential in order to metabolize FG to a compound capable of inducing citrate accumulation, and suggested that the citrate accumulation is associated with the toxic effect.

It remained to be shown that it was the liver and not the alimentary canal that was responsible for the conversion of FG into a substance capable of inducing citrate formation. The simple experiment of removing the canal whilst retaining a normal liver was of course impossible without extensive

Table 4. *Effect of* DL-1-*deoxy*-1-*fluoroglycerol on the urethane-anaesthetized female rat and the influence of functional hepatectomy*

Surgical treatment	Without FG			With FG (85 mg./kg.)		
	Survival (hr.)	Citrate (μg./g.)		Survival (hr.)	Citrate (μg./g.)	
		Heart	Kidney		Heart	Kidney
(*a*) None	3 (killed)	124	175	3 (killed)	755	629
	4 (killed)	157	220	3 (killed)	738	610
	20 (killed)	115	80	33 (died)	960	73
	20 (killed)	64	32	—	—	—
(*b*) Evisceration plus functional hepatectomy	12	51	41	7*	64	29
	12	30	18	13	86	77
				13	27	22
	18	53	115	17	90	38
	20	71	182	—	—	—
(*c*) Evisceration with maintenance of hepatic arterial circulation	4*	125	85	5	122	85
	5*	81	56	—	—	—
	8	90	56	9	125	38
	—	—	—	9	1190	101
	12	204	69	11	2690	121
	—	—	—	12	230	34
	18	104	38	18	108	63
	—	—	—	22†	348	62

* Asphyxiated by tracheal mucus.

† FG 47 mg./kg.

Table 5. *Effect of* DL-1-*deoxy*-1-*fluoroglycerol on the perfused rat-heart–liver preparation*

Addition	Expt.	Heart flow rate (ml./min.) at 0 hr.	1 hr.	2 hr.	3 hr.	Liver flow rate (ml./min.) at 0 hr.	1 hr.	2 hr.	3 hr.	Citrate (μg./g.)* Heart	Liver
None	1	5·0	1·4	0·8	0·6	16	16	8·0	8·0	18	9
	2	5·6	3·0	0·9	—	37	37	35	—	16	25
	3	14·0	8·6	4·3	1·3	11	8·9	12	14	15	6
FG (3 mM)	4	10·9	1·0	—	—	12	9·0	7·2	7·6	45	15
	5	4·9	—	—	—	24	6·0	5·0	4·5	18	20
	6	1·4	1·1	1·3	—	7·3	4·0	8·0	5·0	46	27
	7	7·1	4·0	0·2	—	15	0·6	3·1	4·0	80	25
	8	5·6	4·6	2·5	—	48	48	48	48	123	32
	9	8·9	1·0	—	—	27	19	16	16	61	89
	10	8·6	8·9	6·0	4·4	30	27	27	25	467	251

* Estimated at the end of the experiment.

cannulation, since the principal blood supply to the liver comes from the portal system. However, it was possible to maintain a partial circulation to the liver of the eviscerated animal by retaining the hepatic arterial circulation, which normally contributes about 20 % of the total blood supply to the liver (Markowitz, 1949*b*). One would expect that in a rat treated in this way: (i) if the alimentary canal alone were responsible for the conversion of FG, injected FG would have no effect; (ii) if the liver alone were responsible, injected FG would cause citrate accumulations, but the effect would be far less extensive (by about 80 % perhaps) than in the intact animal.

The results are given in Table 4 (*c*). The control levels of citrate were high, as in Table 4 (*a*); the full evisceration and functional hepatectomy [Table 4 (*b*)] had reduced the elevated citrate levels that urethane produces in the tissues, mainly heart. Elevated citrate levels had previously been noticed with urethane dosing in this Laboratory (R. A. Peters, unpublished work). Thus it seems that the presence of the liver is a prerequisite for this urethane effect. Further, it proves that the technique in Table 4 (*c*) was in fact effectively different from that in Table 4 (*b*). This is relevant to consideration of the results of FG treatment with hepatic arterial maintenance. Out of seven experiments, only three produced clear-cut increases in heart citrate as a result of injecting FG. In two of these three the increases are so very large as to suggest that there was some fundamental difference in the success of their operation compared with the other supposedly identical ones. There is no evidence about what this difference can be. However, the three animals which did show citrate increases were 'eviscerated' and therefore it is certain that the increases can take place in the absence of the alimentary canal and its associated organs.

Perfused heart–liver. The above experiments showed that FG in the 'eviscerated' animals does not cause citrate accumulation in the heart unless the liver is included in the system, and explain the finding that the perfused heart is insensitive to FG. As a corollary, the perfusion of the liver along with a heart should produce citrate accumulation in the heart on addition of FG. This proved to be the case, as Table 5 shows. Considerable citrate increases in the livers were also observed. Substantial accumulations were found only if both the heart and the liver were in good enough condition to be perfused fairly rapidly for over 1 hr. When either was in poor condition, FG induced small citrate accumulation in the heart. If the liver only was in good condition, citrate accumulated in the liver but not in the heart. This suggests that one or more of the systems responsible for metabolizing FG to a citrate-accumulating compound is either in low concentration in the tissue or has a low affinity for FG, and this could account for the failure with preparations of slices or homogenates of mixed liver and kidney to demonstrate the change of FG to the inhibitor of citrate metabolism.

After the heart–liver had been perfused, the solution perfused was collected and its volume reduced upon the steam bath. The resultant solution was potent in inducing citrate accumulation in respiring kidney particles, and most potent in those cases where the heart-citrate levels showed the perfusion to have been most successful. Thus 0·1 ml. of four-times concentrated perfusion solution from Experiment 10 (Table 5) increased the citrate in kidney homogenate by 2·3 μmoles/Warburg flask, that from Expt. 8 increased it by 1·1, and the control (from Expt. 2) by 0·3 μmole. These findings showed that the heart–liver preparation released into the perfusion fluid a 'citrate-accumulating factor'. Experiments were now undertaken to discover if this factor could be fluoroacetate, or other carbon–fluorine substance capable of stopping the metabolism of citrate. It was realised that this would be difficult owing to the small amounts present.

The factor was not removed by passing through a short (2·0 cm. × 0·6 cm.) column of De-Acidite FF, a strong anion-exchange resin; this was found to be due to the presence of phosphate. Eventually a longer column (5·5 cm. × 0·7 cm.) of the anion-exchange resin IRA-400, mesh 100–200, was found to remove both fluoroacetate from a phosphate-containing solution, and citrate-inducing factor from perfusion fluid.

The silica-gel column-chromatographic procedure of Bulen *et al.* (1952) was found to be capable of separating fluoroacetate from other acids. Any fluoroacetate would be eluted by the butanol–chloroform (5:95, v/v) solvent. Concentrated perfusion fluid from Expt. 10 (Table 5) was made alkaline and evaporated to dryness, then taken up in sulphuric acid (as in Bulen *et al.* 1952) and chromatographed in such a system. Fractions (2 ml.) were collected, neutralized, evaporated to dryness, dissolved in buffer and assayed against kidney particles. The results (Fig. 2) showed that a peak of citrate-accumulating activity appeared in the fraction corresponding to the peak for pure fluoroacetate. Fig. 2 represents the second of two similar experiments on the same perfusion fluid. Fluorocitrate is not eluted by butanol–chloroform (5:95, v/v) (P. F. V. Ward & R. A. Peters, unpublished work).

A portion (0·5 ml.) of the four-times concentrated perfusate from Expt. 10, Table 5, also induced citrate accumulation (0·23 μmole) in a pigeon-brain-particle preparation. Such a preparation is insensitive to fluoroacetate (Peters & Wakelin, 1953) but sensitive to fluorocitrate, which may therefore be present in the perfusion fluid, and would contribute to the property of inducing citrate accumulation in kidney particles. Fluorocitrate is exceedingly potent in citrate accumulation in brain particles; 2 μg. (enzymically synthesized)/Warburg flask may give 1 μmole excess of citrate over the control (Peters & Wakelin, 1953). Therefore the above effect of perfusate against brain, if due to fluorocitrate, implies only about 0·2 μg./ml. in the original perfusion fluid. The evidence from the columns is consistent with the presence in the perfusion fluid of fluoroacetate and fluorocitrate. However, other degradation products of FG might be responsible for the observations with brain-particles.

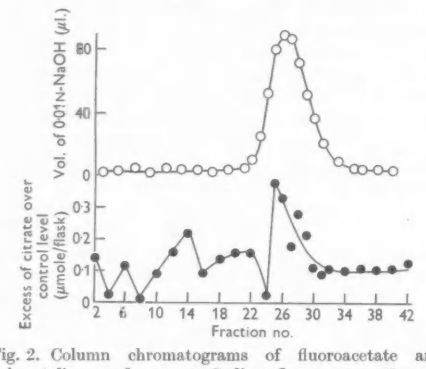

Fig. 2. Column chromatograms of fluoroacetate and heart–liver perfusate. ○, Sodium fluoroacetate (1 mg.); 2 ml. fractions of eluate were titrated with 0·01 N-NaOH; ●, heart–liver perfusate (20 ml.) from Table 5, Expt. 10; 2 ml. fractions were collected and assayed against kidney particles.

Toxicity of epifluorohydrin. Epifluorohydrin is the anhydride of FG. Chemically, hydration of such epoxy compounds is easily achieved by diverse reagents. However, when injected into mice (intraperitoneally, in propylene glycol) the compound was found to have a very low toxicity. Doses up to 100 mg./kg. had no visible effect, nor did they give increased citrate levels in heart or kidney. At 200 mg./kg. death occurred in about 16 hr.: convulsions were not observed (but occasional mild ones could have escaped notice after the first few hours). The animals after 3 hr. were either badly sprawling or prostrate, with lowered body temperatures, and remained more or less prostrate until death. The citrate levels in heart and kidney were not significantly raised in the two animals whose tissues were analysed.

DISCUSSION

The results show that unaltered FG is a metabolically inactive compound, ineffective against all the enzymic systems tested *in vitro*, which included the pathways of glycolysis and of the tricarboxylic acid cycle. Liver slices and homogenate did not convert FG into detectable amounts of a metabolic inhibitor; but the intact liver in a perfused system converted it into a compound which could cause citrate accumulation in the intact heart or in kidney particles. In some experiments also in the 'eviscerated' animal, the liver also caused citrate accumulations from FG, whereas in the absence of the liver no extra citrate was found. The obvious hypothesis is that the compound formed is fluoroacetate or fluorocitrate. The evidence from silica-gel chromatography is that fluoroacetate is present in the perfusion fluid, and the finding with the anion-exchange resin is consistent with this. However, as Fig. 2 shows, there may be other citrate-accumulating factors, eluted by butanol–chloroform (5:95, v/v), in the perfusate. Fluorocitrate may also be present in small amounts. The results of Fig. 2 would be given if the liver produced from FG some compound which in the course of preparation for the column gave rise to fluoro-

acetate. Fluoroacetyl-CoA would give fluoroacetate in these circumstances; in fact even in the mild conditions of the perfusion it would be converted into fluoroacetate to a very large extent (Brady, 1955).

The difference in the behaviour of FG in the slice and in the perfusion is remarkable; it might be thought that it was due to failure to use bicarbonate media, and it should be stressed therefore that these were also used. The simplest explanation would be that there are still unknown co-factors which may escape from slices, but there are other possibilities. In any case, it indicates that there can still be no complacency about negative biochemical results.

As to the pathway of conversion of FG, no evidence is available. A plausible guess would be oxidation (possibly via the aldehyde) to 3-fluoro-3-deoxyglyceric acid (1-fluoro-2-hydroxypropane-3-carboxylic acid), followed by decarboxylation and further oxidation to fluoroacetate. Rudney (1954) showed that propane-1:3-diol could give rise to lactate or to formate; in the latter case a C_2 fragment was presumably formed. If FG followed precisely the same pathway, fluoroacetate would be the C_2 fragment.

The results reported here demonstrate that not only can the animal body perform 'lethal synthesis' (e.g. with fluoroacetate) to transform a compound, which is itself inactive, into a potent anti-metabolite, but that it can also degrade an inactive compound to provide the starting material for a lethal synthesis. The animal is thus prepared to go to some lengths to poison itself.

SUMMARY

1. DL-1-Deoxy-1-fluoroglycerol is toxic to the rat, mouse and guinea pig. Death is associated with convulsions, bradycardia, lowering of body temperature and accumulation of citrate, particularly in heart and kidney.

2. DL-1-Deoxy-1-fluoroglycerol did not affect respiration or citrate level in homogenates or slices of kidney, brain, liver or muscle, nor did it increase the citrate level in the perfused heart.

3. Functional hepatectomy made the rat insensitive to DL-1-deoxy-1-fluoroglycerol.

4. Perfused rat liver converted DL-1-deoxy-1-fluoroglycerol into a compound (or compounds) capable of causing citrate accumulation in a perfused heart or in kidney particles.

5. Chromatography on silica gel of the perfusate from a heart–liver preparation indicated that fluoroacetate was the principal citrate-accumulating factor.

We are indebted to Dr N. F. Taylor and Dr P. W. Kent for providing the DL-1-deoxy-1-fluoroglycerol and the epifluorohydrin; to Dr D. B. Lindsay for advice on perfusion techniques; to Dr P. F. V. Ward for advice on column chromatography, and to Miss P. Ivall for technical assistance. One of us (R.D.O'B.) was in receipt of a Canadian National Research Council Post-Doctoral Fellowship.

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Metabolism of Nicotinamide and Nicotinic Acid in *Neurospora crassa*

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Nicotinic acid is outstanding among the B vitamins in that its metabolic fate has been very extensively investigated. These studies, however, have been carried out mostly in higher animals, and they have revealed that (i) *N'*-methylnicotinamide is, in general, the major end-product of the metabolism of both nicotinic acid and nicotinamide; (ii) nicotinuric acid, the glycine conjugate of nicotinic acid, is also excreted in considerable proportions when nicotinic acid is administered in extraphysiological amounts; (iii) a not insignificant part of the vitamin can be accounted for as respired carbon dioxide; (iv) interconversion of the acid and amide forms of the vitamin does not generally take place on any extensive scale (Roth, Leifer, Hogness & Langham, 1948; Leifer, Roth, Hogness & Corson, 1951; Lin & Johnson, 1953; Reddi & Kodicek, 1953). The study of the metabolism of nicotinic acid and nicotinamide in *Neurospora crassa* was undertaken in this Laboratory in the course of an investigation of the comparative metabolism of the vitamin in different orders of living systems (Sundaram, Radhakrishnamurty & Sarma, 1954; Sundaram & Sarma, 1954, 1956). After a study of the metabolism of the vitamin under growing as well as resting conditions of the mould, it has been possible to demonstrate *in vitro* the presence of an enzyme system capable of deamidating nicotinamide in cell-free extracts of the organism; further, the distribution of this enzymic activity between the fractions produced by differential centrifuging of disintegrated hyphae, as also some of the properties of the enzyme, have been investigated.

The presence of a nicotinamide deamidase was demonstrated for the first time in lactic acid bacteria (Hughes & Williamson, 1953), and two other similar systems have since been reported, both in micro-organisms (Oka, 1954; Halpern & Grossowicz, 1957).

EXPERIMENTAL

Materials

The strains of *N. crassa* used in these studies, Em5297a (wild), 39401 and 3416 (Mitchell, 1950), were maintained by fortnightly subculture on agar slants prepared from Fries's medium (Horowitz & Beadle, 1943) supplemented with 0·2% of yeast extract (Difco) and 0·2% of malt extract (Difco), and 500 μg./100 ml. of nicotinamide with mutants 39401 and 3416. A suspension in sterile water of conidia from a 5- to 6-day-old growth from stock culture was used for all inoculations.

Methods

Metabolism of nicotinic acid and nicotinamide under growing conditions of Neurospora crassa. *N. crassa* was grown at 30° in 250 ml. Erlenmeyer flasks on the basal medium (pH 4·5) supplemented with nicotinic acid or nicotinamide (300 μg./100 ml.). At the end of the required period of growth, the mycelium was removed by filtration and the filtrate was evaporated to dryness *in vacuo* and taken up in a small volume of aq. 50–60% ethanol. After centrifuging, the clear supernatant was analysed for possible metabolites of nicotinic acid and nicotinamide by the ascending paper-chromatographic method (Sundaram *et al.* 1954).

Metabolism of nicotinamide under resting conditions of Neurospora crassa. The procedure adopted was essentially that employed by Yanofsky & Bonner (1951) for the study of the biosynthesis of nicotinic acid by the resting mycelium of *N. crassa*. *N. crassa* was grown for 96 hr. at 30° on 50 ml. lots of the basal medium supplemented with nicotinamide (100 μg./100 ml.). The mycelial pads were filtered off, washed thoroughly with sterile water, pressed dry and used in the following experiment. Three flasks, each containing 20 ml. of sterile water adjusted to pH 4·0–4·2 with dil. HCl, were incubated: one flask with nicotinamide supplement (50 μg.) and mycelium, another with nicotinamide but no mycelium and the third containing mycelium but no nicotinamide, the second and third serving as controls. The flasks were shaken continuously at 30° for 20 hr., at the end of which the mycelia were filtered off and the filtrates analysed as before for nicotinic acid, nicotinamide, nicotinuric acid and *N'*-methylnicotinamide.

Deamidation of nicotinamide by cell-free extract of Neurospora crassa. Mycelial pads of *N. crassa* 39401 grown on the basal medium supplemented with nicotinamide (100 μg./100 ml.) were washed thoroughly with water, and pressed dry and chilled. The chilled mould was thoroughly ground in a mortar surrounded by a freezing mixture of ice and salt, with addition of sand (British Drug Houses Ltd., Poole, Dorset), and homogenized in ice-cold water, so that the homogenate contained the equivalent of 120 mg. of fresh mycelium/ml. The supernatant, obtained after centrifuging of the homogenate at 2000 rev./min. (1000 *g*) for 10 min. to sediment the sand and cell debris, was used as the enzyme source for deamidation of nicotinamide *in vitro*. The complete experimental system contained (in 4 ml.): 1 ml. of 0·2 M-phosphate (Na_2HPO_4–KH_2PO_4) buffer (pH 7·0), 1 ml. of nicotinamide soln. (8 μmoles) and

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2 ml. of the enzyme preparation. In the blank, 1 ml. of water replaced the nicotinamide solution. Incubation was at 37° for 2 hr., at the end of which the ammonia and the free nicotinic acid present in the incubation mixture were determined quantitatively. In preliminary experiments, the deamidation of nicotinamide was inferred by the appearance in the incubation mixture of free nicotinic acid, which was detected by paper chromatography.

Ammonia estimation. The incubation mixture was treated with trichloroacetic acid, final concentration 2·5% (w/v), and centrifuged and the supernatant collected. The residue was washed twice with 2·5% trichloroacetic acid by centrifuging and the washings were added to the main extract. The combined supernatant, after addition of borate buffer (5% borax in 0·5N-NaOH), pH 10·0, till distinctly alkaline to phenolphthalein, was distilled *in vacuo* at 45° in a modified Parnas apparatus (Damodaran, Ramaswamy, Venkatesan, Mahadevan & Ramdass, 1946); the ammonia evolved was absorbed in 0·02N-H_2SO_4 and the solution made up to volume. A portion of the made-up solution was treated with Nessler's reagent and the colour produced measured at 490 mμ with a Lumetron photoelectric colorimeter, model 400A, Photovolt Corp., N.Y., U.S.A.

Free nicotinic acid was determined in the incubation mixture, after heating it for 15 min. in a boiling-water bath, by the microbiological procedure with *Leuconostoc mesenteroides* 9135 (Johnson, 1945), which responds specifically to free nicotinic acid.

Intracellular distribution of nicotinamide deamidase in Neurospora crassa. A homogenate of the mycelia of *N. crassa* was prepared as follows: the washed mycelial pads were pressed dry, then cut to small pieces, and chilled and, after preliminary grinding to a paste (without addition of sand) in a chilled mortar, suspended in isotonic sucrose (0·25M) and immediately passed through a Teflon-pestle homogenizer assembly (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) at 0°. The fractionation of the homogenate into particulate components corresponding to the nuclei, mitochondria and submicroscopic particles of animal tissues and the soluble supernatant was by the low-temperature differential-centrifuging method of Schneider (1948) on an International refrigerated centrifuge (model PR 2). The various fractions thus obtained and a part of the unfractionated homogenate were made up in isotonic sucrose to give in each case a concentration equivalent to approximately 8% (w/v) of fresh *Neurospora* mycelium. The enzyme activities in the various fractions as well as in the whole homogenate were determined by estimating the free nicotinic acid liberated from nicotinamide during an incubation period of 2 hr. as previously described.

Routine estimation of enzyme activity. For routine estimation of enzyme activity, as in the study of the properties of the enzyme and in the survey of the distribution of the enzyme in different strains of *N. crassa*, a 10% (w/v) homogenate of the fresh mycelium was prepared in the Teflon-pestle homogenizer as described above, and the supernatant, after centrifuging at 0° for 1 hr. at 18 000 *g*, served as the enzyme source. The free nicotinic acid released in a 2 ml. system [0·5 ml. of buffer, pH 7·5 (40 ml. of 0·8N-KH_2PO_4 mixed with 17 ml. of 0·8N-NaOH), 0·5 ml. of nicotinamide soln. (4 μmoles) and 1 ml. of enzyme extract] during an incubation period of 2 hr. at 37° was determined microbiologically.

Nitrogen in enzyme extract. Nitrogen in the enzyme extracts was determined by the micro-Kjeldahl method, involving nesslerization of the H_2SO_4–H_2O_2 digest (Koch & McMeekin, 1924).

RESULTS

In the experiments on the metabolism of nicotinic acid and nicotinamide under growing conditions of *N. crassa*, the major metabolite present in the culture medium, as revealed by paper chromatography, was free nicotinic acid, irrespective of whether the medium had been originally supplemented with nicotinic acid or nicotinamide. Neither *N'*-methylnicotinamide nor nicotinuric acid could be detected in any of the culture media. Thus the main feature of the metabolism of the vitamin in the mould is the deamidation of nicotinamide. This capacity of *N. crassa* to convert nicotinamide into nicotinic acid observed under growing conditions was exhibited also under resting conditions of the organism. Here again no other metabolites such as *N'*-methylnicotinamide and nicotinuric acid could be detected in the medium. The data recorded in Table 1 constitute a demonstration of the presence of an enzyme system in *N. crassa* which can convert the amide form of the vitamin into the acid form *in vitro*, and reveal that during this process nicotinic acid and ammonia arise in stoicheiometric proportions, the ratio of ammonia liberated to nicotinic acid released being

Table 1. *Deamidation of nicotinamide by cell-free extract of* Neurospora crassa

At the end of incubation, an amount of nicotinamide was added to the blank equal to that added to the test system before incubation. For experimental details see text.

Time of growth of *N. crassa* (hr.)	Experimental system	Ammonia present in 100 ml. of incubation mixture (μmoles)	Ammonia liberated by deamidation of nicotinamide (μmoles)	Nicotinic acid present in 100 ml. of incubation mixture (μmoles)	Nicotinic acid released by deamidation of nicotinamide (μmoles)	Ammonia liberated / Nicotinic acid released
120	Blank	44·0	21·2	6·5	19·5	1·09
	Test	65·2		26·0		
192	Blank	40·2	17·6	Negligible	14·9	1·18
	Test	57·8		14·9		

Table 2. *Distribution of nicotinamide deamidase activity in the subcellular components of* Neurospora crassa

Incubation system: 1 ml. of 0·2 M-phosphate buffer, pH 7·0, 1 ml. of nicotinamide (8 μmoles) (1 ml. of water in blank), 2 ml. of cell preparation. Incubation was at 37° for 2 hr. Fractions I, II, and III correspond respectively to the nuclei, mitochondria and submicroscopic particles of animal tissues (Schneider, 1948).

Cell fraction	Details of separation	Nicotinic acid released by 2 ml. of cell fraction (μm-moles)	Percentage of total activity in fraction	Nitrogen in 2 ml. of cell fraction (mg.)	Nicotinic acid released/ mg. of N of fraction (μm-moles)
Unfractionated homogenate	—	610	100·0	3·85	158
Fraction I	Precipitate on centrifuging at 600 *g* for 10 min.	94	15·4	1·5	63
Fraction II	Precipitate on centrifuging at 8500 *g* for 10 min.	0	0·0	—	—
Fraction III	Precipitate on centrifuging at 18 000 *g* for 1 hr.	0	0·0	—	—
Fraction IV	Supernatant after centrifuging at 18 000 *g* for 1 hr.	545	89·3	1·34	407

Table 3. *Nicotinamide deamidase in different strains of* Neurospora crassa

The different strains were grown for 72 hr. on basal medium with the appropriate supplement as indicated. Enzyme activity was determined in 1 ml. of supernatant from a 10 % homogenate of the mycelium. The incubation system comprised 0·5 ml. of NaOH–KH_2PO_4 buffer, pH 7·5, 0·5 ml. of nicotinamide soln. (4 μmoles), 1 ml. of enzyme extract. Incubation was at 37° for 2 hr.

N. crassa strain	Supplement to growth medium (500 μg./100 ml.)	Nicotinic acid released in 2 ml. of system (μm-moles)
39401	Nicotinamide	437
Em 5297a (wild)	Nil	595
3416	Nicotinamide	307
39401	Nicotinic acid	545

Table 4. *Stability of nicotinamide deamidase of* Neurospora crassa *at different temperatures*

1 ml. of supernatant from 10 % *N. crassa* homogenate was held at the appropriate temperature for the indicated period of time, at the end of which the enzyme activity was determined as described in Table 3.

Temperature	Time	Destruction of enzyme activity (%)
−20°	72 hr.	28
5	24 hr.	29
5	72 hr.	52
75	6 min.	100
75	3 min.	100
100	5 min.	100
100	2 min.	100

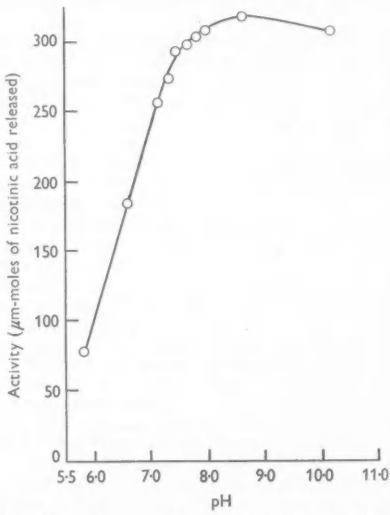

Fig. 1. pH optimum of *Neurospora crassa* nicotinamide deamidase. The enzyme activity in 1 ml. of supernatant from a 10 % homogenate of the mycelium was determined as in Table 3 at different pH values (NaOH–KH_2PO_4 buffer).

very nearly 1:1 on a molar basis. Fractionation of the mycelial homogenate into subcellular components revealed that the nicotinamide deamidase activity is localized almost exclusively in the soluble portion of the cytoplasm (Table 2). The small amount of deamidation associated with the fraction sedimenting at 600 *g* was probably due to the presence therein of a small percentage of intact cells.

The results of further studies on the nicotinamide deamidase of *N. crassa* have been recorded in Tables 3–6 and in Figs. 1 and 2. Evidence for the constitutive nature of the nicotinamide de-

amidase of *N. crassa* was obtained from experiments which showed that the enzyme was present even in the wild strain grown in the absence of nicotinamide or nicotinic acid as well as in strain 39401 grown in the presence of nicotinic acid (Table 3); the enzyme content of the wild strain, in fact, was significantly higher than that of the mutant strains, even though the latter had been grown in the presence of relatively high concentrations of nicotinamide. Data presented in Table 4 indicate that the enzyme is relatively unstable, about 30% of its activity being lost in 24 hr. at 5°. This instability made purification of the enzyme difficult. An acetone-dried powder prepared from the supernatant of a *N. crassa* homogenate was found to retain only 40% of the enzyme activity. The enzyme has a rather broad range of optimum pH (8·5–9·0; Fig. 1). It exhibits maximum activity at about 45° during an incubation period of 2 hr. (Table 5); in view of the rapid inactivation of the enzyme at higher temperatures (Table 4), this result might represent the balance between the activity of the enzyme and its thermal inactivation, and it is possible that the temperature optimum will be higher than 45° if the incubation period is shortened. In Fig. 2 is recorded the activity–substrate concentration relationship of the nicotinamide deamidase of *N. crassa*. In studies on the time course of the enzyme reaction, the deamidation under the conditions of Table 3 was linear up to 100 min.; under the same conditions, the activity increased linearly with enzyme concentration up to 1 ml. of the enzyme extract.

The effects of various compounds and enzyme inhibitors on the nicotinamide deamidase of *N. crassa* are shown in Table 6. The heavy-metal ions Cu^{2+} and Hg^{2+} almost completely inhibited the enzyme; of the rest, only the metal-chelating agents, αα′-dipyridyl, 8-hydroxyquinoline and ethylenediaminetetra-acetate, inhibited the activity to a marked extent, indicating a requirement for metal activator(s) for enzyme action. Although *p*-chloromercuribenzoate lowered the enzyme activity by about 20%, the inability of cysteine to reverse this inhibition and the failure of *o*-iodosobenzoate to inhibit the enzyme do not point to any definite function for –SH grouping in the enzyme action.

Table 5. *Nicotinamide deamidase activity of* Neurospora crassa *at various incubation temperatures*

Enzyme activity in 1 ml. of supernatant from 10% homogenate was determined as described in Table 3 at the temperature indicated.

Temp. of incubation	Nicotinic acid released in 2 ml. of system (μm-moles)
25°	195
32	312
37	454
45	490
52	467
60	316

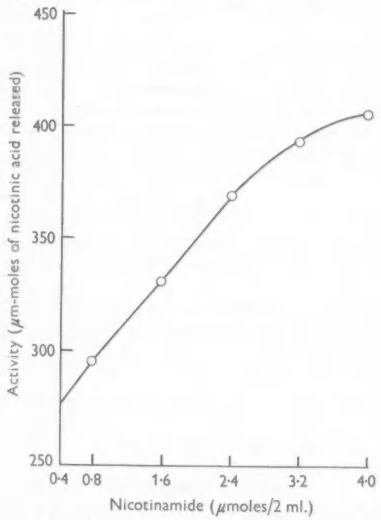

Fig. 2. Effect of substrate concentration on *Neurospora crassa* nicotinamide deamidase activity. The activity in 1 ml. of supernatant from a 10% homogenate of *N. crassa* mycelium at varying substrate concentrations was determined as in Table 3.

Table 6. *Effect of various compounds and inhibitors on nicotinamide deamidase of* Neurospora crassa

The enzyme activity in 1 ml. of supernatant from a 10% homogenate of *N. crassa* was determined in the presence of each of the compounds at the concentration indicated, as described in Table 3.

Compound or inhibitor	Concentration (mM)	Percentage of activity of control
None (control)	—	100·0
Hg^{2+} ions*	1	5·2
Cu^{2+} ions*	1	10·2
Sodium azide	1	95·9
Isoniazid	1	103·5
Isonicotinic acid	1	94·2
Hydrazine	1	92·6
Sodium cyanide	1	95·2
o-Iodosobenzoate	0·01	99·6
p-Chloromercuribenzoate	0·01	80·5
p-Chloromercuribenzoate + mM-cysteine	0·01	88·3
8-Hydroxyquinoline	10	11·3
αα′-Dipyridyl	10	14·7
αα′-Dipyridyl	2	64·3
Ethylenediaminetetra-acetate	10	71·1
Ethylenediaminetetra-acetate	20	61·6

* Incubation mixture was buffered with 2-amino-2-hydroxymethylpropane-1:3-diol.

DISCUSSION

It has been reported earlier (Sundaram *et al.* 1954) that the insect larva *Corcyra cephalonica* St. does not methylate exogenous nicotinamide, but deamidates it to free nicotinic acid, which is thus the principal excretory metabolite of the vitamin in this invertebrate organism. The present investigation demonstrates that *N. crassa* disposes of the vitamin as nicotinic acid and further establishes the presence of the enzyme, nicotinamide deamidase, in the mould. A similar enzyme system has been observed in the *Corcyra* larva (results to be published). *N. crassa* thus appears to resemble the insect larva rather than higher animals like the rat in respect of the metabolism of nicotinic acid and nicotinamide.

In contrast with the methylation of the vitamin and its conjugation with glycine, the deamidation of nicotinamide does not appear to have been recognized as a major metabolic pathway. Investigations which are being carried out in this Laboratory have shown that the tissues of at least two avian organisms, namely the chick and the pigeon, contain nicotinamide deamidase in even greater abundance than either *N. crassa* or *Corcyra*. This would indicate that the deamidation of nicotinamide is not a metabolic reaction confined exclusively to the lower organisms. Relevant in this context is also the fact that birds excrete free nicotinic acid (Komori & Sendju, 1926; Chang & Johnson, 1957), that the pigeon liver is deficient in the enzyme nicotinamide methylkinase, which methylates nicotinamide (Cantoni, 1951), and that the chick does not excrete *N'*-methylnicotinamide as shown by Chang & Johnson (1957) with ^{14}C-labelled nicotinamide. This, taken in conjunction with the results that have been obtained for *N. crassa* and *Corcyra*, suggests that methylation and deamidation of nicotinamide are alternative pathways in the metabolism of the vitamin.

The inability of *N. crassa* to conjugate nicotinic acid with glycine is also striking. It is generally recognized that nicotinuric acid formation is in the nature of a detoxication. Thus the higher animals, such as the rat and man, excrete nicotinuric acid extensively when nicotinic acid is administered in relatively massive amounts (Lin & Johnson, 1953; Reddi & Kodicek, 1953; Sundaram & Sarma, 1956). *Corcyra cephalonica* also excretes nicotinuric acid, to a smaller but significant extent (Sundaram, 1955). It has also been observed in the course of the present studies that nicotinuric acid cannot replace nicotinic acid or nicotinamide for the growth of *N. crassa* 39401, showing that neither does the organism possess the enzyme system capable of splitting the glycine conjugate of the vitamin.

SUMMARY

1. The metabolism of nicotinamide and nicotinic acid has been investigated in *Neurospora crassa*. It has been found that the mould neither methylates the vitamin nor conjugates it with glycine and that the predominant end-product of the vitamin metabolism is free nicotinic acid, even nicotinamide being deamidated.

2. The deamidation of nicotinamide occurs also with *N. crassa* mycelium under resting conditions.

3. The presence of an enzyme capable of releasing free nicotinic acid and ammonia from nicotinamide in stoicheiometric proportions has been demonstrated in cell-free extracts of *N. crassa*.

4. Studies on the fractionation of *N. crassa* into its subcellular components have revealed that the nicotinamide-deamidating activity is localized in the soluble portion of the cytoplasm.

5. The enzyme is constitutive in nature; it is present in the wild as well as mutant strains of the mould, and its formation is independent of the presence of nicotinamide or nicotinic acid in the culture medium.

6. Some properties of the enzyme have been investigated.

7. The comparative metabolism of the vitamin in different orders of living systems and the significance of the deamidation of nicotinamide in this context have been discussed.

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Calcium and Magnesium Metabolism in Calves

2. EFFECT OF DIETARY VITAMIN D AND ULTRAVIOLET IRRADIATION ON MILK-FED CALVES*

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It has previously been reported (Smith, 1957) that calves given a diet consisting almost exclusively of milk with no supplementary vitamin D showed, as they got older, a progressive decrease in the percentage of dietary calcium and magnesium utilized. The addition of sufficient vitamin D to the diet maintained calcium retention at a high level and prevented or cured the hypocalcaemia which frequently also occurred, but it appeared that abnormally large amounts of the vitamin were sometimes required to achieve this. Parr (1957) has also reported hypocalcaemia in milk-fed calves receiving vitamin D in amounts usually considered to be adequate. Part of the object of the present work therefore was to attempt to obtain general confirmation of these results and to examine further the effect of vitamin D levels on calcium utilization in milk-fed calves.

Our previous results (Smith, 1957) suggested that the fall which occurred in magnesium utilization with age and the accompanying hypomagnesaemia were not appreciably influenced by feeding with high levels of vitamin D. Other workers (e.g. Huffman, Conley, Lightfoot & Duncan, 1941; Duncan, Huffman & Robinson, 1935; Parr, 1957) have also demonstrated that milk-fed calves become hypomagnesaemic, usually before 3–4 months of age, and that vitamin D added to the diet does not prevent this. Herman (1936), however, kept eight calves for about 1 year or more on a milk diet with and without vitamin D supplements, and yet reported that no hypomagnesaemia occurred in any of his animals. The only manner in which his treatment appeared to differ appreciably from that of other workers was in the fact that his calves were kept in the sunlight as much as possible. This suggested that ultraviolet-light radiation might produce an effect not shown by the oral administration of vitamin D. A further part of the present work was undertaken in order to examine this possibility.

* Part 1: Smith (1957).

EXPERIMENTAL

Nine bull calves were used. One was an Angus × Ayrshire crossbred (7A), another was an Angus × Shorthorn/Friesian crossbred (11A) and the remainder were Friesians. Apart from the differences described below they received the same general treatment as did the calves used previously (Smith, 1957). They were given 4·40 l. of milk/day up to 10 weeks of age, 6·15 l. of milk/day from 10 to 20 weeks and 8·80 l. of milk/day from 20 weeks. The milk was sampled every day and magnesium and calcium were determined on 4-day batches. The mean magnesium and calcium contents of the batches were 12·5 and 121 mg./100 ml. respectively, measured over the whole experimental period. The standard deviations for individual 4-day batch determinations were ±0·6 mg./100 ml. for magnesium and ±5 mg./100 ml. for calcium. There were no marked changes in milk magnesium from one part of the experimental period to another such as occurred in 1954–55 (Smith, 1957), and it is not therefore considered necessary to give these milk analyses in detail. The calves were given a mineral supplement containing iron, copper and manganese as in the previous work (Smith, 1957). Details of the supplementary vitamin D added to the diet and the ages at which some of the calves were irradiated with u.v. light are shown in Table 1. In addition to the vitamin D supplements, the calves received whatever naturally occurring vitamin D activity was present in their milk diet. No vitamin D assay was carried out on the milk but it is probably valid to use the figures obtained by Henry & Kon (1942). These workers studied milk obtained from the same farm as that from which our milk was obtained. They

found that winter milk, such as our calves received before supplementation with 70 000 i.u. of vitamin D_3/day or u.v.-light treatment, showed an antirachitic activity for rats equivalent to about 10 i.u. of vitamin D/l. For u.v.-light irradiation the calves were shaved on one side and the lamp (Alpine Sun Lamp, model 10, Hanovia Ltd.) was directed at this side. The shaved area was about 3000–4000 cm.^{2}, but part of this area (about 20–40% depending on the calf) was black pigmented. After a milder introductory period of a few days the calves were irradiated for 15 min. daily with the lamp 60 cm. from the skin. This gave a daily dosage of about 10^7 ergs/cm.^{2} of u.v.-light radiation below 3132 Å. Erythema and granular exfoliation occurred but there was not much peeling or blistering, even though the treatment was continued for about 2 months. The treatments with 70 000 i.u. of vitamin D_3/day or with u.v. light were started when the plasma magnesium level was falling, usually when the level was between 1·5 and 1·8 mg./100 ml. This was done in an attempt to use any resultant change in plasma magnesium as an index of greater availability, if any, of dietary magnesium. Balance experiments over periods of 8 days were carried out as previously described (Smith, 1957), usually at approximately monthly intervals, during the whole experimental period. In each of the calves irradiated with u.v. light or given 70 000 i.u. of vitamin D_3/day the last two balance periods reported were carried out after such treatment had started. Four of the calves (nos. 2A, 4A, 7A and 11A) were given diets of magnesium-free synthetic milk (prepared from margarine, casein, glucose, inorganic salts and vitamins) and magnesium-supplemented milk for a period of about 1 month between these last two balance periods, but they continued to get 70 000 i.u. of vitamin D_3/day. Results obtained during this time, which gave information on the amount of endogenous faecal excretion of magnesium, will be reported later. The calves were returned to a normal milk diet at least 8 days before the final balance period.

All the calves remained in general good health, except for calf 14A which lost its appetite and had to be removed from the experiment at 18 weeks of age. There was no diarrhoea during any of the reported balance periods. None of the calves had convulsions or showed other signs of hypomagnesaemia during the reported experimental period, with the exception of calf 2A when it was receiving magnesium-free synthetic milk. The increase in weight of all the calves was regular (except when they were receiving synthetic milk) and generally similar to that found previously (Smith, 1957). The mean weights of the calves were 43 kg. (S.D. ±2·5) at birth, 64·5 kg. (S.D. ±2·5) at 10 weeks, 96·5 kg. (S.D. ±1·5) at 20 weeks and, of the three calves that were kept that long, 142·5 kg. (S.D. ±2·5) at 34 weeks of age.

The methods for determining calcium and magnesium in plasma, urine, faeces and milk were as described previously (Smith, 1957).

RESULTS

Plasma levels

Determinations of plasma magnesium and plasma calcium were made at weekly intervals throughout the experiment. All the calves eventually showed hypomagnesaemia before they were given 70 000 i.u. of vitamin D_3/day or were irradiated with u.v. light. As in previous experiments (see, for example, fig. 2, Smith, 1957) the age of onset of hypomagnesaemia was usually quite distinct. For comparative purposes, when weekly samples are taken, the age of onset of hypomagnesaemia can be defined as that age at which the plasma-magnesium level first shows a negative deviation of more than 15% from the previous mean value, provided that this deviation tends to increase in subsequent determinations. A random deviation of this size was observed on only three occasions in 178 determinations during the initial period during which the plasma-magnesium level was normal. The mean age of onset of hypomagnesaemia, thus defined, was 17 weeks (S.D. ± 1·5). There was no significant difference in this value between calves given 300 or 400 i.u. of vitamin D_3/day and those given no extra supplementary vitamin D. Calves given 300 i.u. of vitamin D_3/day did not show hypocalcaemia when hypomagnesaemia began. Their plasma calcium remained near normal with this supplement for the subsequent 4–5 weeks before they were given a high level of vitamin D_3 or u.v.-light treatment. In accordance with previous

Table 1. *Ages in weeks at which the calves received different amounts of supplementary vitamin* D_3 *or were irradiated with ultraviolet light*

Supplementary vitamin D_3 was added to the milk as an aqueous suspension.

	Treatment				
Calf no.	No supplementary vitamin D	300 i.u. of vitamin D_3/day	400 i.u. of vitamin D_3/day	70 000 i.u. of vitamin D_3/day	u.v. irradiation
1A	0–5	5–15	15–26	—	26–35
2A	0–5	5–15	15–22	22–34	—
4A	0–5	5–15	15–23	23–35	—
7A	0–5	5–15	15–18	18–29	—
8A	0–5	5–15	15–22	—	22–31
11A	0–19	—	—	19–30	—
12A	0–14	—	—	—	14–24
13A	0–25	—	—	—	—
14A	0–18	—	—	—	—

findings (Smith, 1957), calves not given extra vitamin D showed a strong positive correlation between plasma-magnesium and plasma-calcium levels when hypomagnesaemia began. Correlation coefficients, with the numbers of determinations in parentheses, were 0·71 (20), 0·82 (14) and 0·87 (12) for calves 11A, 12A and 14A respectively. Calf 13A showed neither hypomagnesaemia nor hypocalcaemia up to 23 weeks of age and it was slaughtered immediately after the onset of hypomagnesaemia at 24 weeks. As was found previously (Smith, 1957), the addition of a high level of vitamin D to the diet of a hypocalcaemic calf (no. 11A) rapidly restored the calcium level to normal. Irradiation of the hypocalcaemic calf 12A with u.v. light had the same effect (plasma calcium restored from 7 to about 12 mg./100 ml. in 2 weeks).

Neither supplementation with 70 000 i.u. of vitamin D_3/day nor irradiation with u.v. light caused any appreciable improvement in plasma-magnesium levels, and these in fact continued to fall.

Balance experiments

Calcium. The percentages of dietary calcium excreted in the urine and faeces at different ages are shown in Figs. 1 and 2 respectively. The urine excretion was always very low and it was not appreciably affected by vitamin D supplementation or irradiation with u.v. light. In the calves given 300 i.u. of vitamin D_3/day from 5 weeks of age the rate of increase with age in the percentage of dietary calcium excreted in the faeces was less rapid than for the calves not given extra vitamin D. At 6–8 weeks of age, about 2 weeks after the vitamin D supplement was started, their mean calcium retention did not differ significantly from that of the group not given vitamin D, but at 11–13 weeks their mean calcium retention was significantly better ($P < 0{\cdot}02$). At 15–17 weeks the difference between the means was greater but because of the large variance introduced by the high retention shown by calf 13A (72% compared with 27 and 39% respectively for calves 11A and 14A) this difference was not statistically significant. In view of the exceptional resistance of calf 13A to hypomagnesaemia and hypocalcaemia this high retention was also probably exceptional. Determinations on calves 2 weeks or more after their treatment was changed, either by giving them 70 000 i.u. of vitamin D_3/day or by irradiating them with u.v. light, showed a marked improvement in calcium retention, even in calves already receiving 300 i.u. of vitamin D_3/day. It appeared that when either of these treatments was given calves retained calcium with about the same high efficiency at 5 months of age as at 3 weeks of age, but that between 5 and 8 months the retention efficiency decreased slowly.

Magnesium. The percentages of dietary magnesium excreted in the urine and faeces at different ages are represented in Figs. 1 and 3 respectively. The decrease in urine excretion and the increase in faecal excretion of magnesium with age in the present calves were similar to those shown previously (Smith, 1957). There were no significant differences in these changes between calves given 300–400 i.u. of vitamin D_3/day and those given no extra vitamin D. It was also clear that neither supplementation with 70 000 i.u. of vitamin D_3/day nor irradiation with u.v. light had any marked effect on magnesium utilization, although the results obtained did not lend themselves to statistical treatment. There was very considerable variation in the amounts of magnesium retained by the different calves and in general these differences were reflected in the times and rates of onset of hypomagnesaemia. Thus, to take extreme examples, calf 13A which was exceptionally resistant to hypomagnesaemia still retained about 40% of the dietary magnesium and excreted about 8% in the urine at 20–22 weeks of age. Calves 2A and 7A, both of which showed plasma-magnesium values of less than 1·6 mg./100 ml. before 20 weeks of age, retained only about 10% of the dietary magnesium at 20–22 weeks and, in common with other hypomagnesaemic calves, excreted less than 2% in the urine.

DISCUSSION

The general picture of falling efficiency in the utilization of dietary magnesium and calcium with increasing age, and eventual occurrence of correlated falls in plasma-magnesium and plasma-calcium levels, was similar in the present milk-fed calves not given supplementary vitamin D to that observed previously (Smith, 1957). The mean age of onset of hypomagnesaemia (17 weeks, S.D. ± 1·5) in the present nine calves differed significantly ($P < 0{\cdot}01$), however, from the mean age of onset of hypomagnesaemia (11 weeks, S.D. ± 1) in the eight calves given a milk diet without supplementary magnesium and examined in 1954–55 and 1955–56 (Smith, 1957). It is not clear why the present calves were relatively less susceptible to hypomagnesaemia. Treatment, apart from very small differences in the amounts of milk given, was similar and the magnesium content of the milk was not higher in the present experiments. The following facts might be relevant. (*a*) For practical reasons of supply most of the present calves were Friesians, whereas most of the earlier calves were Shorthorns. No difference in the susceptibility to hypomagnesaemia of calves of different breeds has been reported but the matter has not been

adequately studied. (*b*) The calves were kept in unheated pens, except when they were undergoing balance experiments, and were thus subjected to different temperatures depending upon climatic conditions. Hegsted, Vitale & McGrath (1956) have reported that the dietary magnesium requirement of rats may be influenced by environmental temperature. Similar unexplained differences in susceptibility to hypomagnesaemia between groups of calves studied at different times are apparent from an examination of the literature. Thus, for example, despite closely similar treatment the calves of Knoop, Krauss & Hayden (1939) did not show hypomagnesaemia until about 4 months of age whereas those of Parr (1957) began to show hypomagnesaemia in 3–4 weeks.

The finding that magnesium utilization and plasma-magnesium levels, unlike calcium utilization and plasma-calcium levels, were apparently independent of vitamin D intake was also in accordance with previous findings (Smith, 1957). The additional finding that irradiation of some of the calves with u.v. light was also without marked effect on their magnesium utilization, means that no explanation is provided for the absence of

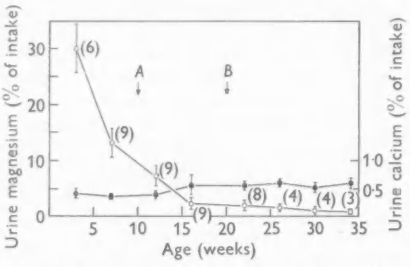

Fig. 1. Effect of age on the excretion of calcium (●) and magnesium (○) in the urine of milk-fed calves. There were no significant differences between the amounts excreted by calves given 300–400 i.u. of vitamin D_3/day and those given no supplementary vitamin D. Similarly, the addition of 70 000 i.u. of vitamin D_3/day or irradiation with u.v. light did not appear to affect the results. Consequently mean values, with standard deviations shown as vertical bars, are given for all the calves irrespective of treatment. The numbers of calves examined, which were the same for both the calcium and magnesium determinations, are shown in parentheses. Experimental points are situated with respect to age in the middle of the 2-weeks age ranges within which the determinations were made. Increases in the amounts of milk fed are indicated as follows: *A*, 4·40–6·15 l./day; *B*, 6·15–8·80 l./day.

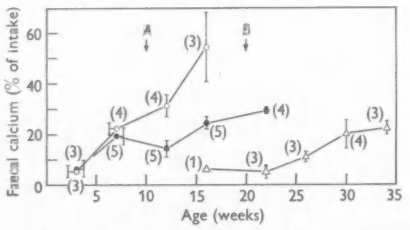

Fig. 2. Effect of age and of different vitamin D supplements on the excretion of calcium in the faeces of milk-fed calves. Treatments were as follows: ○, no supplementary vitamin D; ●, 300 i.u. of vitamin D_3/day from 5 weeks of age and 400 i.u. of vitamin D_3/day from 15 weeks of age; △, 70 000 i.u. of vitamin D_3/day or irradiation with u.v. light. Results for the last two treatments are recorded together since there was no apparent difference between them. Mean values are given with the numbers of calves examined in parentheses and with standard deviations shown as vertical bars. Experimental points are situated with respect to age in the middle of the 2-weeks age ranges within which the determinations were made. Increases in the amounts of milk fed are indicated as follows: *A*, 4·40–6·15 l./day; *B*, 6·15–8·80 l./day.

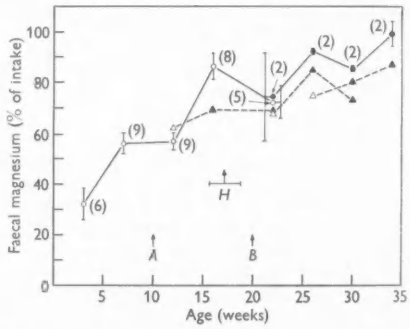

Fig. 3. Effect of age and of different vitamin D supplements on the excretion of magnesium in the faeces of milk-fed calves. Treatments were as follows: ●, 70 000 i.u. of vitamin D_3/day; ○, 300–400 i.u. of vitamin D_3/day or no supplementary vitamin D. Results for the last two treatments are recorded together since there was no significant difference between them. The above results are given as mean values, with the numbers of calves examined in parentheses and with standard deviations shown as vertical bars. The groups of three triangular symbols joined by a broken line each represent results for an individual calf. The first symbol in each group (△) represents a result obtained when the calf was receiving not more than 400 i.u. of vitamin D_3/day. The other symbols (▲) represent results obtained at least 2 weeks after daily u.v.-light irradiation of the calf had been instituted. Experimental points are situated with respect to age in the middle of the 2-weeks age ranges within which the determinations were made. Increases in the amounts of milk fed are indicated as follows: *A*, 4·40–6·15 l./day; *B*, 6·15–8·80 l./day. The arrow *H* indicates the mean age of onset of hypomagnesaemia, with the standard deviation shown as a horizontal bar.

hypomagnesaemia observed in the calves examined by Herman (1936). The results of this worker remain irreconcilable with those of other workers in the field.

According to Bechdel, Hilston, Guerrant & Dutcher (1938) the minimum requirement of normally fed calves for vitamin D is about 6·7 i.u./kg. body wt./day. This figure was, however, derived from experiments in which a diet rather low in phosphorus was given (Ca:P about 3:1). More recently Thomas & Moore (1951), using a better diet in this respect (Ca:P about 1·3–1·8:1), have reported that calves grew normally for 8 months with no blood abnormalities and no signs of rickets when their vitamin D intake was only about 4·8 i.u./kg. body wt./day (less during the first 3 months). Post-mortem examination showed normal bone formation and normal values for bone ash in these animals. Our calves (1A, 2A, 4A, 7A, 8A) which were given 300–400 i.u. of supplementary vitamin D/day from 5 weeks of age received a total intake of vitamin D (or its equivalent as naturally occurring vitamin D activity in the milk) of between about 4·5 and 7·0 i.u./kg. body wt./day. They therefore received dietary vitamin D in amounts which appear to be adequate for normally fed calves. Our results show, however, that this vitamin D intake was not sufficient for optimum calcium utilization in our calves. This regime was not continued for a sufficiently long time to provide evidence on whether any ill effects were potentially associated with the impaired calcium utilization, but hypocalcaemia has been shown in milk-fed calves on even higher vitamin D intakes (Smith, 1957; Parr, 1957). The available evidence favours the view that vitamin D requirements are greater in milk-fed calves than in normally fed calves.

Irradiation of some calves with u.v. light appeared to produce an effect on calcium metabolism similar to that produced by giving vitamin D in large amounts (70 000 i.u./day).

SUMMARY

1. The addition of a supplement of 300–400 i.u. of vitamin D_3/day to the diet of milk-fed calves reduced the rate of decrease in calcium utilization as the calves got older and prevented the plasma-calcium level from falling when the plasma-magnesium level fell.

2. The addition to the diet of 70 000 i.u. of vitamin D_3/day or irradiation of the calves with u.v. light greatly improved the utilization of calcium both in calves previously receiving no vitamin D and in those receiving 300–400 i.u. of vitamin D_3/day.

3. The mean faecal excretion of magnesium increased from 32% of the dietary magnesium at about 3 weeks of age to 86% at about 16 weeks of age, after which it did not change greatly. Neither the addition of vitamin D to the diet nor irradiation of the calves with u.v. light had any appreciable effect on this excretion.

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The Inhibition of Mitosis by the Reaction of Maleic Hydrazide with Sulphydryl Groups

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Maleic hydrazide, or 6-hydroxy-2*H*-pyridazin-3-one, inhibits the growth of plants (Leopold & Klein, 1951) and has been used to control the sprouting of stored potatoes and onions. McLeish (1953) demonstrated that the site of action of the chemical was on the chromosomes of the *Vicia* cell, so affecting the early stages of mitotic division. Studies on the effect of maleic hydrazide on the enzymes of intact cells suggest that maleic hydrazide affects the enzymes requiring sulphydryl (SH) groups for activity (Andrae & Andrae, 1953; Bertossi, 1955; Muir & Hansch, 1953; Isenberg, Odland, Prop & Jensen, 1951). No reaction, however, between maleic hydrazide and soluble SH

groups has been demonstrated *in vitro* at physiological pH values (Leopold & Price, 1956) or in the presence of tissue extracts (Weller, Ball & Sell, 1957). These findings differ from the known reactions between other maleic acid derivatives and the SH group present in glutathione (GSH) and proteins. These reactions result in the addition of the SH group to the double bond of the maleic acid (Morgan & Friedman, 1938).

The present study is an attempt to bring some consistency to these contrasting results by comparing the reactions of maleic hydrazide and SH compounds *in vivo* and *in vitro* and also to obtain information on the biochemical mechanism of mitosis. The germinating pea seed was chosen as experimental plant material since S. P. Spragg & E. W. Yemm (unpublished work) have shown that considerable changes in glutathione occur when this seed germinates. Analyses for the GSH in the intact seeds and in the separated axes (plumules and radicles) were combined with a study of inhibition of enzymes by maleic hydrazide. Inhibition of division was found to be associated with interference with SH metabolism.

MATERIALS AND METHODS

Treatment of the pea seeds. Pea seeds of the variety Meteor were used in all the experiments. The procedure used for treating the seeds was to soak these for 48 hr. at 20° and then to transfer them to moist soil. The time of first wetting the seeds was taken as zero time for the experiments. Under these conditions between 90 and 100% of seeds soaked in water germinated normally.

Maleic hydrazide solution. Pure maleic hydrazide was dissolved in the appropriate buffer solutions for the enzyme reactions, and in water for treating the seeds. These solutions were found to be stable for at least 1 month at room temperatures.

p-*Chloromercuribenzoate.* An approx. 4 mM-solution was obtained by dissolving the acid in water and adjusting the pH with N-NaOH to the value used in the experiment.

Glutathione. A commercial preparation was used; iodate titrations showed that the sample contained at least 90% of GSH. *N*-Ethylmaleimide, glucose 1-phosphate (dipotassium salt), hexose diphosphate (disodium salt) and casein were commercial preparations.

Determination of colour intensity. In all the determinations the intensity of the colours was measured with the EEL portable colorimeter (Evans Electroselenium Co. Ltd., Harlow, Essex).

Buffer solutions. The acid buffer used was 0·2M-sodium acetate buffer adjusted to pH 5·4; the neutral buffer was a 0·1M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) adjusted to pH 7·4. The pH of the enzyme digests did not alter during the reaction.

Estimation of glutathione and oxidized glutathione. GSH and oxidized glutathione (GSSG) were extracted by grinding the seeds with cold 2·5% (w/v) sulphosalicylic acid, following the method described by S. P. Spragg & E. W. Yemm (unpublished work). The nitroprusside method of Grunert & Phillips (1951) was used to estimate the GSH, by measuring the intensity of the red colour (Ilford filter no. 624). The method is not specific for GSH since other SH compounds will react with the nitroprusside. However, Hopkins & Morgan (1943) isolated GSH from freshly germinated pea seeds and S. P. Spragg & E. W. Yemm (unpublished work) showed that the predominating soluble SH compounds in germinating Meteor pea seeds were GSH and GSSG. In view of these results the soluble SH compounds of the cotyledons and the axes have been called GSH and GSSG. Total glutathione was determined by repeating the above estimation on a second sample of the extract after electrolytic reduction of the GSSG by the method of Dohan & Woodward (1939). The values for GSH and GSSG did not vary more than ±10% from the mean of duplicate extractions.

Estimation of β-amylase activity. A commercial preparation of β-amylase was incubated with soluble starch under the conditions given in the legend to Fig. 1. The reducing sugar produced by the hydrolysis of the starch was estimated by the Somogyi copper method (Somogyi, 1945). This method gave results repeatable to ±5% of the mean of duplicate samples each containing 100 μg. of glucose.

Preparation and estimation of the activity of potato-starch phosphorylase. A partially purified solution of potato-starch phosphorylase was prepared and its activity was determined by the procedures described by Hanes (1940). The enzymic conversion of glucose 1-phosphate into starch was carried out in 10 ml. centrifuge tubes, and after incubation of the mixtures (conditions and concentrations are given in the legend to Fig. 2) the enzyme was inactivated by the addition to the tubes of an equal volume of 10% (w/v) trichloroacetic acid. The precipitates were removed by centrifuging and the free inorganic phosphate in the supernatant solutions was estimated by the method of Peel & Loughman (1957). The blue colour produced was measured by means of the Ilford red filter no. 608. Estimations of phosphatase activity were obtained by determining the inorganic phosphate liberated from the glucose 1-phosphate in the absence of soluble starch. In all the preparations of the enzyme used, the phosphatase activity was negligible compared with that of the phosphorylase enzyme. The method yielded results repeatable to within ±10% of the mean of duplicate determinations of activity.

Preparation and estimation of activity of aldolase. A partially purified preparation of aldolase was isolated from germinating pea seeds by ammonium sulphate precipitations by means of the initial steps in the procedure described by Stumpf (1948). The activity was determined by the method of Beck (1955). The resultant colour intensity was measured with the yellow-green Ilford filter no. 625. Enzyme blanks were prepared by adding the hexose diphosphate to the solution after precipitation of the enzyme.

Estimation of the activity of trypsin. A volume (0·3 ml.) of 0·01% solution of crystalline trypsin (L. Light and Co., Colnbrook, Bucks) was treated at 32° with 3 ml. of casein solution (1% in 0·1M-tris, pH 7·0) and 4 ml. of 10 mM-MH in 0·1M-tris, pH 7·0, or water. Samples of the reaction mixture were removed at intervals and the enzyme was inactivated by the addition of an equal volume of 10% trichloroacetic acid. After centrifuging, the free amino acids in the supernatant were determined by the method of Yemm & Cocking (1955). The intensity of the blue colour was measured with the aid of the Ilford yellow-green filter

no. 625. This method gave results repeatable to within ±5% of the mean duplicate estimations.

Estimation of protein SH *groups.* The SH groups of β-amylase were estimated by titration with 0·01 N-$AgNO_3$, by the amperometric method of Benesch, Lardy & Benesch (1955).

RESULTS

Inhibition of growth of pea seedlings

During the early stages of germination of the pea seed maleic hydrazide had no visible effects on the emergence of the radicle from the enclosing testa. As the radicle enlarged, the inhibitory effects of mM-maleic hydrazide became apparent, and after 4–5 days the treated seedlings stopped growing. This inhibition of growth appeared to be permanent since no further growth occurred when the seedlings were left for a further 20 days in the soil. At the 4- to 5-day stage, the tip of the treated radicle appeared rounded and slightly bulbous in shape, the hypocotyl was swollen and, if the plumule did emerge, the small leaflets were pointed and did not increase in size. These treated seedlings differed markedly from the control seedlings where normal growth was associated with a long slender radicle tapering to a point. Microscopical examination of the cells of the growing point of treated seedlings showed that very few of the cells were dividing. Furthermore, the few cells which were dividing had mitotic figures characteristic of the early stages of mitotic division. This state contrasted sharply with that in the controls where cells were present in all stages of division. Examination of developing pea seedlings showed that the initial growth of the radicle was mainly by cell enlargement, and maleic hydrazide had no visible effects on this process.

Table 1 shows the results of one experiment illustrating the effect of maleic hydrazide on the amounts of GSH and GSSG in the intact seeds during the first 42 hr. of germination. Germination was accompanied by an increase in the amount of GSH in both the treated and control seeds and a decrease in the quantity of GSSG. Relatively small quantitative differences were found between the two sets of seeds in that more GSH was detected in the seeds treated with maleic hydrazide.

In one experiment the seeds were treated with different concentrations of maleic hydrazide and estimates were obtained of the amounts of GSH and GSSG in the axes after 4 and 10 days of growth, and the results are shown in Table 2. After 4 days, the quantity of GSSG/unit fresh weight ranged from 160 μg. in the water control to zero in mM-maleic hydrazide. With one exception, the decrease in GSSG was relatively uniform as the maleic hydrazide concentrations were increased.

Table 1. *Effects of* 10 mM-*maleic hydrazide on the reduced and oxidized glutathione in germinating pea seeds*

Time (hr.)	Treated (μg./seed)		Control (μg./seed)		100 × Treated/control	
	GSH	GSSG	GSH	GSSG	GSH	GSSG
16	130	78	154	60	84·4	130
42	220	52	191	47	115	110

Table 2. *Effects of different concentrations of maleic hydrazide on the content of reduced and oxidized glutathione and the germination of pea seedlings*

Seeds were soaked for 48 hr. and then planted. Analyses were made on the plumule plus radicle only.

Concn. (mM) ...	1·0	0·66	0·33	0·1	Control
		(μg./g. fresh weight)			
		After 4 days			
GSH	567	510	640	410	476
GSSG	0·0	83	209	120	166
Total	567	593	849	530	642
Total $\frac{GSH}{GSSG}$	∞	6·17	3·2	3·4	2·87
		After 10 days			
GSH	256	211	182	195	164
GSSG	73	78	123	133	100
Total	329	289	305	328	264
Total $\frac{GSH}{GSSG}$	3·5	2·7	1·48	1·48	1·64
% Germination	25·0*	30·0*	48·0*	89·0	86·0

* Seedlings did not appear normal at 10 days and did not grow above 5 cm. high.

In contrast, the amount of GSH increased as the concentration of maleic hydrazide increased. The high figures for GSH and GSSG in the seedlings treated with 0·33 mM-maleic hydrazide were caused by an abnormally low fresh weight in this sample. The relationship between GSH and GSSG at varying concentrations of maleic hydrazide was emphasized by expressing the results as the ratio GSH:GSSG. This ratio increased steadily as the concentration of maleic hydrazide increased. The absence of GSSG in the samples treated with maleic hydrazide at the 4-day stage of growth was confirmed by several experiments. In general, the amount of GSH was higher in the treated seedlings than in the controls.

A further sample of seedlings was analysed after 10 days, and a similar though less marked effect of maleic hydrazide on the ratio GSH:GSSG was found. Slightly more total glutathione was detected in the treated seedlings than in the control. This effect was probably the result of comparing seedlings growing normally with those whose growth had been arrested, for it has been found that a decrease in the total GSH found is associated with normal growth. The percentage germination was assessed and it was most noticeable that this increased with decreasing concentration of maleic hydrazide and, hence, with increasing amounts of GSSG, although normal growth of the seedling after this stage was found only in the 0·1 mM-solution.

Summarizing, the major effect of maleic hydrazide on the glutathione of the dividing cells of the axis was to disturb the equilibrium between GSH and GSSG, so leading to an accumulation of GSH in the tissues. Comparison of these effects with the results found for seeds in the early stage of germination, where the equilibrium was unaffected, shows that glutathione functions in at least two differing systems, one of which is important to mitotic division.

Inhibition of enzymes with maleic hydrazide

The results of the analyses on growing tissue show that maleic hydrazide interferes in SH metabolism, although no direct reaction has been demonstrated between the soluble GSH compounds and maleic hydrazide *in vitro* (Weller *et al.* 1957). The following experiments show that enzymes requiring SH groups for activity are inhibited by maleic hydrazide in an irreversible manner.

The effect of maleic hydrazide on β-amylase, an enzyme requiring free SH groups for activity (Weill & Caldwell, 1945), is shown in Fig. 1. The progress curves for the action of the enzyme on soluble starch show that in the presence of maleic hydrazide the amount of reducing sugar liberated in 15 min. at pH 5·0 was approx. 53 % of the control. Similarly, at pH 7·3 the amount of sugar liberated was 61 % of the control in the same time (this figure was obtained from an experiment separate from that quoted in Fig. 1). These inhibitory effects were confirmed in other experiments, where it was found that the inhibition increased if the enzyme solution was stored for a day or more at 5°. These storage conditions did not materially affect the rates of sugar production for the controls and the explanation of this change is not known.

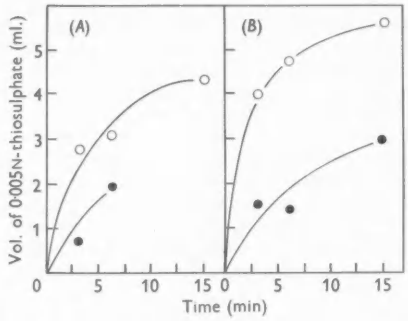


Fig. 1. Progress curves of β-amylase in the presence of maleic hydrazide (●) and water control (○). pH values of the digest were 7·8 (*A*) and 5·0 (*B*). A volume (0·5 ml.) of enzyme (1 μg./ml.) was incubated at 32° with 2 ml. of buffer [(*A*) 0·1 M-tris; (*B*) 0·2 M-acetate] and either 12·5 ml. of water or 6·5 ml. of water plus 6 ml. of 10 mM-maleic hydrazide.

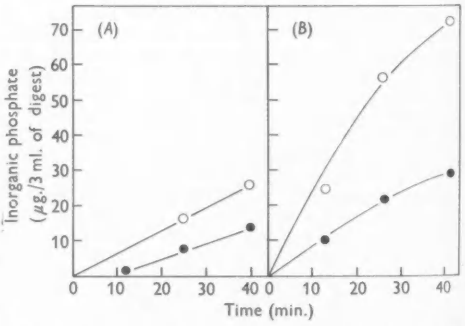


Fig. 2. Progress curves of starch phosphorylase in the presence of maleic hydrazide (●) and water (○). pH values of the digests were 7·4 (*A*) and 5·2 (*B*). A volume (0·5 ml.) of enzyme (11·5 mg. of N/ml.) was incubated at 32° with 0·1 ml. of soluble starch (1 %), 0·3 ml. of buffer [(*A*), 0·1 M-tris; (*B*) 0·2 M-acetate] and 1 ml. of either 10 mM-maleic hydrazide or water. The reaction was started by the addition of 0·1 ml. of glucose 1-phosphate (46 mg./ml., dipotassium salt).

Further evidence for maleic hydrazide inhibition through inactivation of SH groups on the protein chain was obtained with starch phosphorylase, an enzyme which also requires the presence of free SH groups for activity (Elliot, 1946). The progress curves for the liberation of phosphate from glucose 1-phosphate as a result of starch synthesis are shown in Fig. 2. As with β-amylase, the activity of the enzyme was reduced by maleic hydrazide at the two pH values studies. After 48 min. the phosphate liberated in the presence of maleic hydrazide was 54 and 41 % of the controls at pH 7·4 and 5·8 respectively. For comparative purposes the activities of the same enzyme preparation were inhibited with *p*-chloromercuribenzoate and *N*-ethylmaleimide and the results are shown in Table 3. Both *p*-chloromercuribenzoate and maleic hydrazide were more active inhibitors than *N*-ethylmaleimide, but at equal molar concentrations maleic hydrazide was not so effective an inhibitor as *p*-chloromercuribenzoate for this enzyme. The phosphatase activity of the enzyme preparations remained low and unaffected by the maleic hydrazide.

Sulphydryl enzymes can be protected against the action of *p*-chloromercuribenzoate or iodoacetamide by GSH or cysteine. Attempts were made therefore to decrease the maleic hydrazide inhibition of starch phosphorylase by the inclusion of GSH in the reaction mixture. The concentration of GSH used was sufficient to decrease the effective concentration of maleic hydrazide by half if any reaction occurred between the two chemicals. Tests had shown that if the maleic hydrazide was decreased to this amount its inhibition of this enzyme preparation was small. The results given in Table 4 (with a different enzyme preparation from the previous experiments) show that GSH does not affect the maleic hydrazide inhibition at either pH value used. These results are in agreement with the previous data which show that maleic hydrazide inhibition of growth occurs even in the presence of excess of GSH *in vivo* (Table 2).

The effect of maleic hydrazide on the activity of two further enzymes was studied. The two examined, trypsin and pea aldolase, contrasted with the previous enzyme in that they do not require free SH groups on the protein for activity. The activity of trypsin, however, is dependent on the presence of S·S groups on the protein (Liener, 1957). Previous investigators have demonstrated the presence of SH groups on purified aldolase proteins (Benesch *et al.* 1955), but since *p*-chloromercuribenzoate did not inhibit the crude enzyme preparation these are not essential for activity. Maleic hydrazide did not inhibit the activity of aldolase or trypsin under the conditions used.

Table 3. *Inhibition of potato-starch phosphorylase by N-ethylmaleimide, maleic hydrazide and p-chloromercuribenzoate*

A volume (0·5 ml.) of enzyme (11·5 mg. of N/ml.) was incubated at 32° with 0·1 ml. of 1 % soluble starch, 0·3 ml. of 0·1 M-tris buffer, pH 7·4, 0·1 ml. of glucose 1-phosphate (46 mg./ml., dipotassium salt) and 1 ml. of either 10 mM-maleic hydrazide or 10 mM-*N*-ethylmaleimide, or 5 mM-*p*-chloromercuribenzoate or water. Results have been corrected for blanks.

	μg. of P/3 ml./15 min.	Inhibited (%)
Control	18·9	—
N-Ethylmaleimide	13·9	73·5
Maleic hydrazide	10·0	53·5
p-Chloromercuribenzoate	9·7	51·0

Table 4. *Inhibition of potato-starch phosphorylase by maleic hydrazide in the presence of glutathione*

A volume (0·5 ml.) of enzyme was incubated at 32° with 0·1 ml. of 1 % soluble starch, 0·3 ml. of 0·1 M-tris buffer or 0·2 M-acetate buffer, 0·1 ml. of glucose 1-phosphate (46 mg./ml., dipotassium salt), 0·5 ml. of GSH (3·3 mg./ml.) and 1 ml. of 10 mM-maleic hydrazide or water. Results have been corrected for blanks.

	μg. of P/3 ml./15 min.	
	pH 7·4	pH 5·4
Maleic hydrazide + GSH	8·0	11·6
Control	14·7	23·7
$100\left(\frac{\text{Maleic hydrazide + GSH}}{\text{Control}}\right)$	54·3	48·2

These experiments suggest that maleic hydrazide inhibits enzymes requiring free SH groups for activity, but more detailed experiments are necessary to explain the mechanism of this inhibition. The inability of maleic hydrazide to inhibit trypsin shows that maleic hydrazide cannot destroy the S·S grouping present on the protein chain.

Effect of maleic hydrazide on the SH *groups of amylase*

Further evidence that protein SH groups and maleic hydrazide combine was obtained by titrating the SH groups remaining on β-amylase protein after treatment with maleic hydrazide. Two digests, one with and one without maleic hydrazide, were incubated under the experimental conditions of Fig. 1 and, after 90 min., 2 ml. samples were taken. The enzyme protein was denatured with 8 M-urea, following the conditions given by Benesch *et al.* (1955), and the free SH groups were titrated with 0·01 N-$AgNO_3$, by an amperometric method. Treatment with urea was necessary since the control sample showed no uptake of Ag^+ ions before denaturation. However, the treated sample, which gave the same titre before and after denaturation, was found to contain only 40 % of the free

SH groups found in the control sample (typical figures being 0·43 μM-SH/2 ml. for the control sample compared with 0·18 μM-SH/2 ml. for the maleic hydrazide-treated sample).

Maleic hydrazide-treated and untreated β-amylase solutions were incubated for 90 min. at 27° with GSH (concentration of GSH was 2·5 mM) and 2 ml. samples titrated. To prevent the interference of GSH in the titration of the protein SH groups, 75% of the GSH was removed by the addition of *N*-ethylmaleimide to both the treated and untreated samples. After denaturation with urea the untreated protein SH was 0·55 μM-SH/2 ml., compared with 0·2 μM-SH/2 ml. for the treated protein. However, after dialysis of both the treated and untreated protein solutions against distilled water for 18 hr. at 5°, the protein SH groups were the same. Thus dialysis appears to remove maleic hydrazide from the SH groups on the protein chain. The significance of this result is not clear but it seems possible that the maleic hydrazide is removed by a mass-action effect.

Similar experiments with starch phosphorylase (Table 4) suggested that the inhibition by maleic hydrazide was not reversed by GSH and more direct evidence for this was found by amperometric titration.

Ultraviolet absorption of maleic hydrazide and maleic anhydride

Qualitative tests with bromine water and permanganate solutions showed that at least one of the double bonds in the molecule is ethylenic in character and thus similar to the double bond in maleic anhydride.

The u.v. light-absorption spectra of maleic hydrazide shows that the molecule is stable in the enolic form (6-hydroxy-2*H*-pyridazin-3-one) (Miller & White, 1956). These results have been confirmed and show that in 0·1 N-HCl, maleic hydrazide has two absorption maxima, one at less than 210 mμ and the second at 300 mμ (Fig. 3). These maxima shifted in 0·07 N-NaOH to 219 and 330 mμ respectively. In contrast, maleic anhydride has only one maximum below 210 mμ in 0·1 N-HCl, and at 220 mμ in 0·07 N-NaOH, so corresponding with one of the maxima in the maleic hydrazide absorption curve. In equal molar concentrations maleic hydrazide absorbed more strongly than the anhydride at 210 mμ in acid solution.

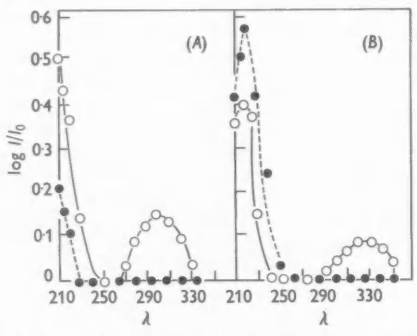


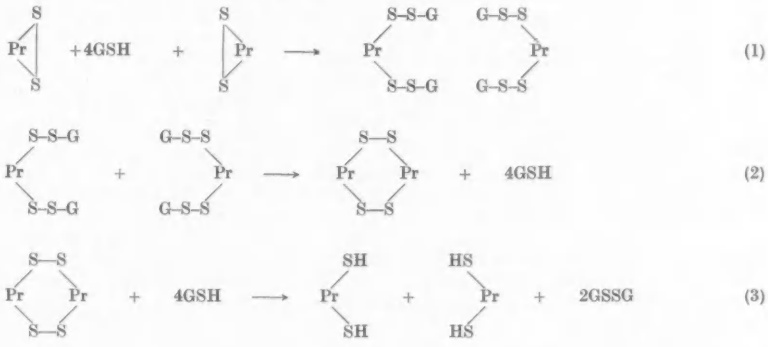
Fig. 3. U.v. light-absorption curves of maleic hydrazide (○) and maleic anhydride (●). (*A*), Solvent 0·1 N-HCl, $\lambda_{max.}$ for maleic hydrazide at 300 mμ; (*B*), solvent 0·07 N-NaOH, $\lambda_{max.}$ for maleic hydrazide at 218 mμ and 320 mμ; $\lambda_{max.}$ for maleic anhydride at 220 mμ.

The presence of one maximum common to both the anhydride and maleic hydrazide, together with the marked reactivity of maleic hydrazide to the double-bond reagents, suggest that the double bond between the carbon atoms of maleic hydrazide possesses a certain proportion of the high reactivity characteristic of maleic acid derivatives. This reactivity is not sufficient to allow maleic hydrazide to condense with SH groups of glutathione and other soluble thiol compounds in the manner of other maleic acid derivatives.

DISCUSSION

S. P. Spragg & E. W. Yemm (unpublished work) have shown that in the early stages of germination of pea seeds there occurs a conversion of GSSG into GSH. This change in the redox equilibrium of glutathione was not affected by maleic hydrazide, showing that in intact tissue maleic hydrazide and GSH do not interact. It is also inferred from these results that maleic hydrazide has negligible effect on the functioning of the enzyme systems associated with the oxidation and reduction of glutathione (Mapson & Goddard, 1951; Mapson & Moustafa, 1956).

The growth of the radicle in the germinating seeds can be divided into two stages. The first is the emergence of the radicle through the testa and is mainly the result of cell enlargement of pre-existing cells; the second follows with mitotic division contributing to a major proportion of the growth. Maleic hydrazide did not affect the cell enlargement, but influenced the equilibrium between GSSG and GSH during division. Furthermore, the ability of high concentrations of maleic hydrazide to effect a complete conversion of GSSG into GSH in the dividing cells suggests that during mitosis in the treated tissue either there is an increase in the activity of the enzyme systems reducing glutathione relative to the activity of those oxidizing it or that other reactions which specifically require glutathione are inhibited. As the axis aged there was a partial recovery of the equilibrium between the two redox forms.



These results indicate that, in addition to its many known biochemical reactions, glutathione participates in reactions specific to cells preparing to divide. In view of the reaction between maleic hydrazide and the SH groups on proteins it appears that the additional role involves a reaction between glutathione and protein SH groups, the maleic hydrazide intervening and preventing normal functioning of this reaction. The effect of varying the concentration of maleic hydrazide (Table 2) is of interest in relation to the importance of the glutathione–protein SH cycle, since the continued growth of the axis was found to be associated with the maintenance of the GSH:GSSG ratio.

Mazia (1954) has summarized the evidence of previous investigators, which shows that GSH is intimately involved in the preliminary stages of mitotic division of sea-urchin eggs, and he has suggested that the reactions (1) and (2) above (in which Pr represents protein) occur during the division of the fertilized egg.

This sequence of reactions points to the importance of glutathione and protein SH groups in mitotic division in animal cells. The present results show that important reactions also occur between SH groups in the dividing plant cell. The fact that GSH accumulates at the expense of the GSSG in the maleic hydrazide-treated cells (Table 2) suggests that the maleic hydrazide has blocked a process which in the normal cell reoxidizes GSH. In view of this evidence it seems possible that reaction (3) may represent the process of oxidation.

In a comparative study of a non-dividing mutant of *Candida albicans*, Nickerson & Falcone (1956) found that division of the fungal cell was associated with the presence of an enzyme which reduces protein disulphide groups. It seems possible that reaction 3 may be catalysed by a similar type of enzyme. The dynamic state of this process is dependent on the continual re-formation of GSH from GSSG. This conversion may take place by reduction with reduced triphosphopyridine nucleotide (Mapson & Goddard, 1951), although no definite evidence is available to show that this compound is the only one capable of reducing GSSG in the dividing cell.

The structural similarities which were found to exist between maleic hydrazide and maleic anhydride suggest that the maleic hydrazide may form an addition compound with some SH groups in the protein molecule, possibly before the oxidation step, and this prevents the subsequent breaking of any disulphide groups which may have formed during the preliminary steps of division. In fact, the experiments with maleic hydrazide suggest that, in plant cells preparing to divide, the maintenance of certain protein SH groups is essential for subsequent normal mitotic division.

Finally, McLeish (1953) has shown that maleic hydrazide can produce chromosome aberrations, and it is of interest to speculate on the mechanisms involved in this process and their relationships with SH groups during mitosis. A reaction could take place between two SH groups and one molecule of maleic hydrazide in such a manner that two protein chains become linked irreversibly. In the subsequent migration of the proteins to form the two new cells, the chains joined by maleic hydrazide must either move as a unit, or split so that each new molecule consists of proteins of the old molecule condensed via the maleic hydrazide. In either case the genetic constitution may be affected by an unbalanced movement of the proteins and the formation of the unnatural links between the peptide chains. On this basis part of the normal course of division would be the controlled formation of the S·S bonds between specific proteins followed by reduction of these bonds by GSH.

SUMMARY

1. Pea seeds treated with maleic hydrazide germinated normally until cell enlargement was replaced by cell division, when growth stopped.

2. The ratio of reduced (GSH) to oxidized (GSSG) glutathione, i.e. GSH:GSSG, in cotyledons was not affected by maleic hydrazide but at the onset of cell division GSH increased at the expense of GSSG in treated axes only.

3. Certain enzymes requiring free sulphydryl groups (starch phosphorylase and β-amylase) were inhibited irreversibly by maleic hydrazide, but other enzymes (trypsin and pea aldolase) were not affected.

4. The results suggest that maleic hydrazide reacts with protein SH groups and thus inhibits the reduction of protein S·S by GSH during mitosis.

We are indebted to Mr H. Holland for the microscopical examinations of the radicle tips and to Dr F. Haworth for helpful discussions during the course of the work.

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Formation of Antibody by Isolated Perfused Lungs of Immunized Rabbits

THE USE OF [^{14}C]AMINO ACIDS TO STUDY THE DYNAMICS OF ANTIBODY SECRETION

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Under the usual experimental conditions yields of newly formed proteins secreted by tissue slices are too small to permit their isolation for study without carrier protein, and when whole organs, such as the liver, are perfused with blood (e.g. Miller, Bly, Watson & Bale, 1951) recovery of any newly synthesized plasma proteins is complicated by admixture with similar proteins already present in the perfusion fluid.

Normal lung forms little or no γ-globulin, nor is it known to secrete any other protein. When rabbits are hyperimmunized by intravenous injections of a particulate antigen, such as pneumococci type 3, the lung may become a major site for antibody formation, as shown both *in vivo* (Humphrey & Sulitzeanu, 1958) and by tissue slices *in vitro* (Askonas & Humphrey, 1958). As a result of hyperimmunization there is enormous proliferation of lymphoid tissue and plasma cells around the small arteries and the lung may increase two to four times in weight. If such lungs are perfused with salt medium containing normal

rabbit serum, antibody is secreted and can be specifically precipitated from the perfusing fluids by adding the appropriate antigen. A considerable net synthesis both of antibody (up to 8 mg./hr.) and of other γ-globulin can be demonstrated.

For a study of protein synthesis this system has, however, two disadvantages common to other perfused organs. The first is that tissues contain several kinds of cells, and the second that it is impossible to wash out all preformed plasma proteins from extracellular spaces so as to prevent their mixing with proteins newly formed during the experiment. The second difficulty can be overcome by injecting into the animal, 1 or 2 days before death, specific antibody labelled with ^{131}I, as described by Humphrey & Sulitzeanu (1958). Such antibody equilibrates with the proteins in the extracellular fluids, and if it is assumed that at the time of death the ^{131}I specific activities of antibody in the plasma and tissue fluids are the same, the proportion of preformed antibody in samples isolated during the experiment can easily be calculated.

In the experiments described below the incorporation of [^{14}C]amino acids into intracellular and extracellular antibody was followed, and the specific radioactivity of intracellular and extracellular free amino acids was studied during the perfusion. The bearing of our findings on the secretion of antibody and other proteins is discussed. By secretion we mean the release from within the cell into the surrounding medium of protein derived from the intracellular pool, but not necessarily newly formed during the experimental period. Evidence is also given that the lung from an immune animal may be a site of plasma-protein breakdown.

EXPERIMENTAL

Immunization procedure. Sandylop rabbits (2·5–2·8 kg.), bred at the N.I.M.R., were given intravenous injections of pneumococci type 3 killed with formalin or of alum-precipitated ovalbumin or both. Injections were made on alternate days for 3–4 weeks, the quantity of antigen injected being gradually increased up to 10^{10} pneumococci or 0·5 mg. of ovalbumin. The animals were test-bled at intervals. Antibody estimations were made by precipitating with antigen in the equivalence zone, followed by estimation of γ-globulin in the washed precipitates by dissolving in 0·1 N-NaOH and measuring the light absorption at 280 mμ.

Administration of [^{131}I]*antibody.* γ-Globulin concentrates were prepared by fractionation with 16% (w/v) Na_2SO_4 of sera from sandylop rabbits hyperimmunized against pneumococci type 3. Of the protein in the preparations, 70–75% was specifically precipitable with pneumococci type 3 capsular polysaccharide, prepared by the method of Heidelberger, Kendall & Scherp (1936). The globulin concentrates were iodinated with ^{131}I by the jet-mixing method of McFarlane (1956), so as to contain not more than 1 g.atom of I/mole of protein. About 100 μc of [^{131}I]protein (5–16 mg.) was injected intravenously 2 days before the experiment. By precipitating antibody from a serum sample taken at the time of death and measuring its γ-ray radioactivity, the ^{131}I specific radioactivity of preformed extracellular antibody was determined.

Perfusion of the isolated lung. After removal of a preliminary blood sample from the rabbit 500 i.u. of heparin followed by 120 mg. of sodium pentobarbitone were injected intravenously. The heart and lungs were carefully removed and rinsed, and one cannula was inserted into the trachea and another into the pulmonary artery; through the latter 500 ml. of cold balanced-salt solution (Hanks, 1948) was perfused to wash out most of the blood. After trimming away most of the heart and loose tissue, the lung was suspended in the apparatus illustrated in Fig. 1.

The apparatus, for whose design we are indebted to Mr W. E. Brocklehurst, consisted essentially of a jacketed vessel and a pump for recirculating a small volume of perfusion fluid at 39·5°. The lung was alternately inflated and deflated with O_2+CO_2 (95:5) gas mixture by means of a respiration pump (Palmer and Co., Brixton). In some experiments the surface of the lung, especially at the bases, was scratched gently with a sharp needle in order to diminish accumulation of fluid which sometimes occurred after perfusion for some hours. In most experiments this was not necessary.

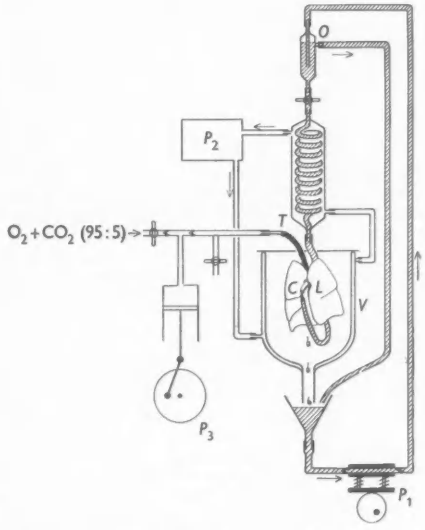


Fig. 1. Arrangement of apparatus for perfusion of rabbit lung. *L*, Lung; P_1, small-volume force pump for recirculating about 50 ml. of medium through the lung; P_2, pump circulating water from a bath at 39·5°; P_3, respiration pump supplying O_2+CO_2 (95:5) (stroke volume 50 ml.); *T*, cannulated trachea; *O*, overflow for perfusate; *C*, cannula in pulmonary artery; *V*, jacketed vessel.

The perfusion mixture consisted of 90 ml. of isotonic salt medium (Hanks, 1948), containing 0·27 g. of glucose, 5000 units of sodium penicillin, 1–3 μc of [^{14}C]glycine, L-[^{14}C]lysine or L-[^{14}C]valine, a complete amino acid mixture (15 mg. of N) prepared from acid-hydrolysed plasma protein with added L-tryptophan and 10 ml. of fresh normal rabbit serum. In experiments where the specific radioactivity of the free valine was determined, the medium contained known amounts of all the L-amino acids (15 mg. of N/100 ml. of medium). The rabbit serum was obtained by collecting blood without anticoagulant into chilled silicone-treated centrifuge tubes (Silicone Fluid M.S. 200/350 cs., Hopkin and Williams Ltd.), and centrifuging the cells and platelets in the cold, and transferring the plasma to glass tubes and allowing it to clot spontaneously. This was done to avoid the high levels of histamine and 5-hydroxytryptamine, which occur in fresh clotted rabbit serum as a result of platelet destruction during clotting and can cause initial pulmonary vasoconstriction.

The lung was perfused with a known volume (about 50 ml.) of fluid at about 30 ml./min. In most experiments the complete medium, but without [^{14}C]amino acid, was circulated for about 30 min. and was then replaced by labelled medium; after this the perfusion fluid was collected and replaced by new medium every 60–90 min. to maintain the level of radioactivity and of amino acids. To make it easier to judge when one lot of medium had been completely replaced by fresh, and to assess how effectively the lung was perfused, two drops of 5% Pontamine Sky Blue (6 Bx) were added to alternate changes of medium.

Radioactive amino acids. Biosynthetic [^{14}C]glycine, L-[^{14}C]lysine and L-[^{14}C]valine were obtained from the Radiochemical Centre, Amersham, Bucks, each with a specific activity of about 55 μc/mg.

Isolation of antibody and γ-globulin from perfusates. About 50 mg. of the appropriate inactive amino acid was added to each lot of perfusing fluid, to dilute the radioactive amino acid and decrease non-specific adsorption on to proteins. The perfusates were dialysed under pressure to a volume of 8–10 ml. against 0·6% NaCl solution containing 66 mM-sodium phosphate buffer, pH 7·5. Any sediment was removed by centrifuging for 15 min. at 15 000 *g*, and more inactive amino acid (30–50 mg.) and Merthiolate (Eli Lilly and Co.) to a concentration of 0·1 mM were added to the supernatant fluid.

Specific antibody was then precipitated by addition of an appropriate amount of antigen. A rough idea of the quantity to be added could be obtained by determining the amount of extracellular antibody present by measurement of the ^{131}I. Since extracellular antibody usually accounted for less than half of the antibody isolated, enough antigen was added in the first instance to precipitate twice the estimated extracellular antibody. The mixture was incubated for 20 min. at 37° and left overnight in the cold room. Antigen–antibody precipitate was centrifuged down at low speed (about 3 min. at 1600 *g*). A little more antigen was added to the supernatant and the process repeated until no more precipitate was obtained. The pooled precipitates were washed thrice with 0·9% NaCl solution, once with aq. 50% (v/v) ethanol, twice with ethanol, and dried with ether and weighed. Radioactivity due to ^{131}I and ^{14}C was determined as described below.

A sample of the remaining γ-globulin could be isolated from the perfusing fluid after removal of the specific antigen–antibody precipitates by successive precipitations with Na_2SO_4 at concentrations of 18 and 16% (w/v). The fraction soluble in 0·9% NaCl after dialysis against water was freeze-dried. This fraction contained only γ-globulin as tested by paper electrophoresis. It included both γ-globulin secreted by the lung and the normal rabbit γ-globulin added to the perfusion medium. However, any ^{14}C radioactivity must have been due to γ-globulin newly formed from [^{14}C]amino acids in the medium.

Isolation of intracellular antibody from lung. In some cases one lobe of the lung was tied off and removed part way through the experiment and intracellular antibody was isolated from this portion as described below. At the end of each experiment, except when stated otherwise, the lung was perfused for about 3 min. with Hanks's solution without added amino acids, and weighed and frozen at −30°. The frozen lung was weighed, chopped and extracted in a high-speed blender at 0° with 5 ml. of normal rabbit serum and about thrice its weight of saline phosphate buffer, pH 7·5, containing 50 mg. of inactive amino acid. The material sedimenting in 10 min. at 1600 *g* was re-extracted with about half the previous quantity of saline phosphate buffer, and the combined extracts were centrifuged for 20 min. at 22 000 *g*. The opalescent supernatant may be expected to contain a representative sample of intracellular lung antibody, although complete extraction can hardly be claimed. After pressure dialysis of the extract against saline phosphate buffer to reduce its volume to about 20 ml., the pH was adjusted to 5·8 in order to aggregate finely suspended particles and some of the nucleoprotein. After centrifuging, the clear supernatant was neutralized and antibody was isolated as described for the perfusion fluids above. Specific radioactivities due to ^{131}I and ^{14}C were measured on the pooled antigen–antibody precipitates, since it had been found in early experiments that the specific radioactivities of the first and second precipitates in any one sample did not differ significantly.

Radioactivity of free valine in the perfusate and in cells of the lung. The lung was perfused with 60 ml. of medium containing 6 ml. of normal rabbit serum, 50 mg. of glutamine and 43 mg. of amino acids, of which 3·4 mg. was L-valine, including 1·8 μc of L-[^{14}C]valine (18·3 μg./μc). The medium was changed every 80–90 min. during a total period of 5½ hr. Samples (10 ml.) were taken from the original medium, and from the perfusing fluids 10 min. after the beginning and at the end of each perfusion period, and added to equal volumes of 20% (w/v) trichloroacetic acid; precipitated protein was removed, the supernatant was freed from trichloroacetic acid by repeated extractions with ether and its volume reduced to 2–3 ml. by evaporation. After neutralization, the samples were desalted on a column of Zeo-Karb 225 in the H^+ form (1 cm. × 4·5 cm.). The sample was washed through the column with 30 ml. of water, and the amino acids were eluted with aq. 2N-NH_3 soln. and taken to dryness over H_2SO_4 in a desiccator. About a quarter of the material was applied on chromatography paper (Whatman no. 3) along a line 4 cm. long, and the chromatogram developed for 48 hr. with redistilled collidine–lutidine–water (1:1:2, by vol.). Marker valine was spotted 4 cm. away from each sample. A strip containing the marker valine and a small part of the sample was cut out, and the location of the valine was determined by spraying with ninhydrin reagent. Valine was then eluted

from the unsprayed papers with aq. 50% (v/v) ethanol, and transferred to 2 cm.² polythene disks, and dried and its radioactivity determined. The valine was next eluted from the disks with 3 ml. of water and the amount determined by the ninhydrin method (Jacobs, 1956). Similar-sized pieces of blank chromatographic paper were treated in the same way, and the valine values corrected for the traces of ninhydrin-positive material present in the paper. Specific radioactivities were expressed as counts/min./100 μg. of valine. The error in this method was found to be about 15% by carrying out determinations on valine with known specific radioactivity.

Intracellular free valine was extracted within 5 sec. of stopping the perfusion by freezing the lung in liquid air, and weighing it and grinding it with 2–3 ml. of 20% (w/v) trichloroacetic acid for each gram of tissue. Isolation of the valine from the trichloroacetic acid extracts and determination of its radioactivity were carried out as described above. [^{14}C]Valine was isolated also from the intracellular- and extracellular-antibody precipitates after hydrolysis in 6N-HCl for 24 hr. at 100° by the method described above.

Radioactivity determinations. ^{131}I radioactivities were determined in a well-type scintillation counter, with 3 ml. samples of liquids, or with a known weight of dried protein in the form of a pellet at the bottom of a small pointed tube for antibody or non-specific γ-globulin precipitates. The counter efficiency was about 25% in each case, and the background was about 400 counts/min.

[^{14}C]Amino acids were plated on 2 cm.² polythene disks and counted at infinite thinness under a Geiger–Müller counter with a mica end-window (1·7 mg./cm.²). [^{14}C]-Proteins, in the absence of ^{131}I, and $BaCO_3$ were counted at infinite thickness on 0·3 or 1 cm.² disks, and their specific radioactivities expressed as counts/min./cm.².

When ^{131}I was also present, the radioactivity due to ^{14}C was measured in a proportional counter after combustion to CO_2 (Bradley, Holloway & McFarlane, 1954). Antibody specific radioactivities were not corrected for the presence of antigen, which formed 4 and 10% (w/w) of the C in precipitates of antibodies to pneumococci and ovalbumin respectively. In any one experiment, only specific radioactivities obtained by a given method were compared with one another.

Measurement of γ-globulin breakdown during perfusion. The perfusates collected at different time intervals and a sample of the rabbit's serum were pressure-dialysed overnight against 400 ml. of phosphate saline buffer at 3°. The diffusates were evaporated to a small volume, after adjustment of the pH to about 9·5, and the ^{131}I content of the diffusible and non-diffusible material was determined. Increases in the percentage of diffusible ^{131}I above that found in the serum sample were taken to represent protein breakdown.

RESULTS

Synthesis of antibodies and other γ-globulin. Two typical experiments are described. In Expt. 1 the lung was obtained from a rabbit hyperimmunized by intravenous injections of formalin-killed pneumococci type 3, the last injection having been given 2 days before death. A few minutes after the last injection of antigen 100 μc of ^{131}I-labelled rabbit γ-globulin (75% antibody specifically precipitable with capsular polysaccharide) was also administered to enable us later to recognize preformed extracellular antibody by its ^{131}I-label.

The lung, which was three to four times normal weight, was perfused with unlabelled medium for 30 min.; the fluid was then collected and replaced with a fresh lot containing 2 μc of L-[^{14}C]lysine; this process was repeated after 120, 195 and 290 min. After 190 min. one lobe of the lung was tied off and removed for extraction of antibody, and the remainder of the lung was extracted at the end of the experiment. Antibody was also isolated from the perfusates, and the specific radioactivities due to ^{131}I and ^{14}C were measured. Table 1 shows the weights of antibody actually isolated, and the proportion of preformed extracellular antibody. It is striking that antibody isolated after perfusion for

Table 1. *Amount of intracellular and extracellular antibody isolated during perfusion of lung from a rabbit hyperimmunized against pneumococci type* 3

Two days before killing, 100 μc of [^{131}I]-labelled globulin concentrate containing 75% of specifically precipitable antibody was injected intravenously. The percentage of extracellular antibody is calculated by comparing the ^{131}I specific activity of antibody samples with that of the serum antibody at death. Antibody content of serum at death: 43 mg./ml. One lobe of lung was removed at 195 min.

	Time of perfusion (min.)	Amount of antibody precipitate isolated (mg.)	Extracellular antibody (%)	Amount of antibody secreted by lung (mg.)
Perfusates	0–30	13	83	2·2
	30–120	24·6	56	10·8
	120–195	17·8	48	9·3
	195–290	13·9	40	11·2*
	Total	69·3		33·5
Lung lobe (14 g.) at 195 min.		<14	1·6	—
Remainder of lung (38 g.) at 290 min.		29	1·9	—

* Corrected for lobe removed at 195 min.

4 hr. still contained 40 % of preformed extracellular antibody. A total of 69·3 mg. of antibody was isolated during perfusion for 5 hr., and of this 33·5 mg. was newly secreted by the lung. The rate of secretion (about 7 mg./hr.) was uniform over the whole period of perfusion (Fig. 2). This is probably an underestimate since some losses are apt to occur during pressure dialysis and isolation of the antibody.

The ^{14}C specific radioactivity of newly secreted antibody in the perfusate increased steadily after the first 90 min. Estimations at earlier times in several other experiments showed that ^{14}C never appeared in secreted antibody in less than 30 min., and significant labelling usually did not occur for up to 1 hr.

γ-Globulin was also isolated, though not quantitatively, from the perfusion fluids after removal of all precipitable antibody. Each lot of medium contained about 60 mg. of γ-globulin from the added normal serum, and any γ-globulin secreted by the lung was therefore diluted to an unknown extent. However, since considerable incorporation of [^{14}C]lysine occurred, the lung must also have been synthesizing and secreting γ-globulin other than specific antibody. If the specific radioactivity of secreted γ-globulin is assumed to be equal to that of antibody secreted at the same time, the amount secreted during the second perfusion period (between 120 and 195 min.) would have been 15 mg. During this period 9·3 mg. of antibody was also secreted, making a secretion rate of about 18 mg. of total γ-globulin/hr. The nature of such non-specific γ-globulin has been discussed by Askonas & Humphrey (1958).

In this experiment the antibody extracted from the lung after 190 and 290 min. had almost the same ^{14}C specific radioactivity as antibody isolated from the perfusing fluid at the same time. However, this was not always true, as is shown in the next experiment.

In Expt. 2 the rabbit had received eleven intravenous injections of pneumococci and eight of alum-precipitated ovalbumin during the preceding 4 weeks. Serum-antibody levels had declined by about 30 % during the last week, indicating that antibody synthesis had slowed down markedly. At death the serum contained 14 mg. of anti-capsular polysaccharide and 2 mg. of antiovalbumin/ml. The lungs were greatly enlarged.

Perfusion was carried out as above with medium containing 8 μc of [^{14}C]glycine, and the two antibodies were isolated successively by specific precipitation with antigen. The results are summarized in Fig. 3. The two antibodies had similar specific radioactivities, although the amount of antibody against capsular polysaccharide secreted in 5 hr.

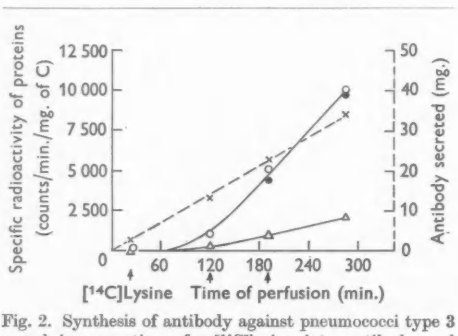


Fig. 2. Synthesis of antibody against pneumococci type 3 and incorporation of L-[^{14}C]lysine into antibody and other γ-globulin by lung from hyperimmune rabbit. The lung was perfused according to the schedule in Table 1; arrows indicate the times of addition of complete medium containing 2 μc of L-[^{14}C]lysine. Radioactivity due to ^{14}C was determined with a proportional gas counter and expressed as counts/min./mg. of C. The radioactivity measurements were corrected for preformed extracellular antibody. ×, Antibody precipitate (mg.) isolated from perfusate (corrected for preformed extracellular antibody); ○, ^{14}C specific radioactivity of antibody precipitate; △, ^{14}C specific radioactivity of residual γ-globulin isolated from perfusates; ●, specific radioactivity of intracellular antibody from lung after 195 and 290 min.

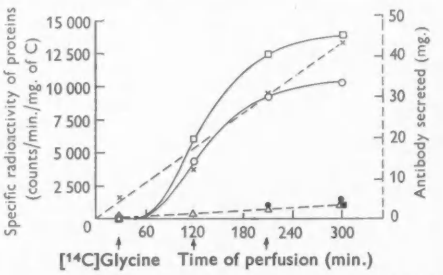


Fig. 3. Synthesis of antibody and incorporation of [^{14}C]-glycine during perfusion of lung from rabbit hyperimmunized against pneumococci type 3 and ovalbumin. Arrows indicate the times at which fresh lots of complete medium containing 8 μc of [^{14}C]glycine were added. Radioactivity due to ^{14}C was determined with a proportional gas counter and expressed as counts/min./mg. of C. The radioactivity measurements were corrected for preformed extracellular antibody. ×, Antibody (mg.) against capsular polysaccharide isolated from perfusates (corrected for preformed extracellular antibody); ○, ^{14}C specific radioactivity of antibody against capsular polysaccharide; ●, ^{14}C specific radioactivity of intracellular antibody against pneumococcal capsular polysaccharide; □, ^{14}C specific radioactivity of antiovalbumin; ■, ^{14}C specific radioactivity of intracellular antiovalbumin; △, mg. of antiovalbumin (cumulative) isolated from perfusing fluids.

Table 2. *Comparison of specific radioactivities of secreted antibody and of antibody isolated from the lungs after perfusion with [^{14}C]amino acids*

Specific radioactivity of antibody was determined by gas counting, and the values were corrected for the presence of preformed extracellular antibody

Expt.	Radioactive amino acid used	Perfusion time (min.)	Specific radioactivity (counts/min./mg. of C) Antibody from perfusate	Specific radioactivity (counts/min./mg. of C) Antibody from lung	Total antibody secreted (mg.)	Wt. of lung after perfusion (g.)	Serum-antibody content (mg./ml.) At death	Serum-antibody content (mg./ml.) Week before death
A	Glycine (7 μc)	230	39 000	8245	—	—	7·2	12
		340	78 000	8200	7·2	38	—	—
B	L-Lysine (1·75 μc)	340	10 200	9700	4·5	20	4·9	5
C	L-Lysine (1·25 μc)	325	5 000	4170	5·2	20	4·3	2·9
D	L-Lysine (1·75 μc)	300	10 200	5850	12	31	10	12·2
E	Glycine (8 μc)	210	9 300	957	—	—	—	—
		300	10 400	1562	43	74	14·6	19·7
F	L-Lysine (2 μc)	195	5 200	4400	—	—	—	—
		290	10 000	9850	33·5	52	43	29

(43 mg.) was much more than that of antiovalbumin (approx. 3·4 mg.). Forty-eight hours before death the rabbit had received ^{131}I-labelled anticapsular polysaccharide, but no [^{131}I]antiovalbumin. The radioactivities of both antibodies were corrected for the presence of preformed extracellular antibody on the basis of measurement of ^{131}I-labelled antibody against capsular polysaccharide. With antiovalbumin the correction is only approximate because the relative proportion of the two antibodies secreted by the lung is not necessarily the same as their proportion in serum.

In this experiment the ^{14}C specific radioactivities of both types of intracellular antibodies were only 11–17 % of the specific radioactivities of the antibody secreted into the perfusion fluid at the corresponding times.

Comparison of specific radioactivity of intracellular and secreted antibody

Six typical experiments are summarized in Table 2, and show how the findings varied when we compared the specific radioactivities of intracellular antibody (taken at the end of the experiment) and of antibody secreted during the last perfusion period. In each case the lung perfused was from a rabbit immunized by intravenous injections of pneumococci. In half of the experiments the specific radioactivities of the intracellular and secreted antibody were very similar. In the other half intracellular antibody had a very much lower specific radioactivity, 10–50 % of that of the antibody secreted into the perfusate; in these experiments the antibody content of the serum was declining during the days before the experiment. A possible interpretation of these findings will be discussed below.

Table 3. *Specific radioactivity of free valine during perfusion*

Fresh lots of perfusing medium were added after 85, 162 and 240 min.

Material	Time of perfusion (min.)	Specific radioactivity of isolated valine (counts/min./100 μg.)	Relative radioactivity of free valine (%)
Medium*	0	8630	—
Perfusate*	10	4870	—
Perfusate*	85	1900	—
Perfusate*	95	5550	—
Perfusate*	162	5300	—
Perfusate*	172	7820	—
Perfusate*	240	5640	—
Perfusate*	250	6840	—
Perfusate*	330	6125	100
Lung*	330	3500	57
Antibody† (in perfusate)	240–330	550	9
Intracellular lung antibody†	330	630	10

* Free valine was isolated from trichloroacetic acid filtrates.

† Valine was isolated from antibody hydrolysates.

Radioactivity of intracellular and extracellular free valine and antibody valine during perfusion

L-[^{14}C]Valine was chosen for this study because valine is not converted into any other amino acid by the cells and is relatively easy to isolate. The lung of an immune rabbit was perfused with complete medium (containing 1·8 μc of L-[^{14}C]-valine); this was replaced by fresh medium after 85, 162 and 240 min. Free [^{14}C]valine was isolated from 10 % trichloroacetic acid filtrates of the perfusates at the beginning and end of each perfusion period, and from the lung at the end of the experiment. The results may be seen in Table 3. During

the first 10 min. the specific radioactivity of the free valine fell from 8630 counts/min./100 μg. of valine to 4870, by almost half its original value. Since the medium contained 3·4 mg. of L-valine, the valine in the medium must have been diluted by an almost equal amount of intracellular free valine from the 27 g. of lung. Lung contains about 14% of protein (Spector, 1956); if we assume the protein to contain 5% of valine, an intracellular pool of almost 3·5 mg. would represent 2% of the total protein-bound valine. As perfusion proceeded, the specific radioactivity of the valine fell further, and after 85 min. was one-fifth of the original value; this suggests continuous breakdown of unlabelled lung protein to free amino acid in the cells, but only a small percentage of the total lung protein can be involved. When the medium was replaced after 85, 162 and 240 min. with fresh medium containing valine with a specific activity of 8630 counts/min./100 μg., the specific radioactivity of the valine fell during each perfusion period to about two-thirds of its original level. Hence a small amount of breakdown of lung protein to freely exchangeable valine continues during the entire period of perfusion. The use of Pontamine Sky Blue in the medium showed that all of the lung was being perfused. The intracellular free valine of the lung at the end of the experiment had a specific radioactivity of 3500, i.e. 57% of the radioactivity of the extracellular free valine at the same time.

[^{14}C]Valine was also isolated from the antibody secreted by the lung (corrected for the presence of extracellular ^{131}I-labelled antibody) and from intracellular lung antibody (Table 3). It was found to have a specific radioactivity about one-fifth of that of the intracellular free valine isolated from the lung at the end of the experiment. The radioactivity of the total lung protein, precipitated with trichloroacetic acid, was also determined; over 18% of the L-[^{14}C]valine added to the perfusing fluid was incorporated into the protein of the lung. More than half of this radioactivity was associated with the protein readily extractable with isotonic NaCl from the lung after breaking the cells.

Two other confirmatory experiments were carried out, one with L-[^{14}C]valine and the other with amino acids obtained by acid hydrolysis of algal [^{14}C]protein. In the latter experiment the specific radioactivity of the α-amino carboxyl group was determined as $BaCO_3$ after release of the CO_2 by ninhydrin (Van Slyke, McFadyen & Hamilton, 1941), as described by Humphrey & Sulitzeanu (1958). In both these experiments, similar decreases in specific radioactivity of the free amino acid during the perfusion period were observed. As in the experiment described above, the intracellular free amino acid in the lung had a radioactivity about two-thirds of that of the extracellular amino acid. Although the specific radioactivity of the antibody secreted into the perfusate during the last hour was almost the same as that of the intracellular antibody, the specific radioactivity of the ^{14}C of the α-amino carboxyl group of the constituent amino acids was less than one-fifth of that of the free amino acids in the lung.

Breakdown of ^{131}I-labelled proteins by the lung during perfusion

When ^{14}C- and ^{131}I-labelled proteins of the same kind are administered simultaneously to animals, they are catabolized at practically identical rates (Cohen, Holloway, Matthews & McFarlane, 1956; McFarlane, 1956). Perfusion of ^{131}I-labelled proteins through the isolated liver results in splitting off of non-protein iodine only when protein breakdown occurs (Gordon, 1957). There are good grounds therefore for considering that liberation of non-protein iodine by the lung from ^{131}I-labelled plasma protein indicates catabolism of the proteins. In our experiments the rabbits had received ^{131}I-labelled γ-globulin 48 hr. before the lung perfusion, and a small fraction of the dose was retained in the interstitial fluids of the lung. During the perfusion part of the ^{131}I was split off, the proportion of diffusible iodine in the perfusates being three to five times greater than that in the serum of the same rabbit taken at the time of death. This implies that γ-globulin was being broken down and that, if there was complete mixing of ^{131}I-labelled γ-globulin and the serum proteins in the perfusates, the lung would have been breaking down during each hour about 5% of the γ-globulin in the medium (about 70 mg.), i.e. up to 3·5 mg. of γ-globulin/hr. To confirm this finding, the lung from a hyperimmunized rabbit, which had not received ^{131}I-labelled protein *in vivo*, was perfused with medium containing 0·1 vol. of normal serum to which had been added a few milligrams of ^{131}I-labelled γ-globulin. Under these

Table 4. *Catabolism of ^{131}I-labelled γ-globulin by hyperimmunized lung*

The lung of a hyperimmunized rabbit was perfused with 60 ml. of medium containing 6 ml. of normal rabbit serum and 3 mg. of rabbit γ-[^{131}I]globulin. The medium was changed after 35, 110, 190 and 290 min. (All counts were at least three times the background count.)

Perfusion period (min.)	Diffusible ^{131}I in perfusate (%)	Recovery of ^{131}I counts in perfusate (%)
Original medium	2·3	—
0–35	5·5	80
35–110	5·8	97
110–190	6·2	78
190–290	5·9	100

conditions also splitting off of non-protein ^{131}I occurred and to a similar extent (Table 4). Since only γ-globulin was labelled it is not possible to state whether lung also catabolized other plasma proteins.

DISCUSSION

Lungs from hyperimmunized rabbits have been perfused and have been shown to synthesize and secrete specific antibody and other γ-globulin at a rate comparable with that found for plasma proteins by livers perfused with blood (Miller *et al.* 1951).

Although such lungs are not obtained as readily as normal livers, they have one great advantage, namely that antibody can easily be isolated from the perfusion medium, and that accurate allowance can be made for any preformed extracellular protein which was present at the beginning of the experiment. Such correction is necessary since about 40 % of the antibody appearing in the perfusate was not newly secreted even after perfusing for 2–3 hr. We have calculated the efficiency of conversion of the labelled valine into antibody by the lung in an experiment in which the levels of free valine were determined in all samples of medium. During this experiment about 30 mg. of antibody was newly formed in 5 hr. A total of 4 μc of valine was added. Of this 0·92 μc was recovered as free valine in the samples of medium removed and 0·072 μc was recovered in antibody isolated from the perfusates and from the lung at the end of the perfusion. Thus of the 3·08 μc which apparently entered the lung tissue, 0·07 μc (about 2·3 %) was converted into antibody. If we assume that the amount of other γ-globulin formed was 1·5 times the amount of antibody (see Expt. 1 above), the total converted into secretable globulins was 5·7 %. This is almost certainly an underestimate, since the recovery of free valine and of antibody was probably not complete. In intact rabbits the percentage of administered [^{14}C]amino acids which are incorporated into plasma proteins varies between about 3 and 9 %, depending on which amino acids are used (J. H. Humphrey & A. S. McFarlane, unpublished work).

The radioactivity found in the lung protein (after extraction with trichloroacetic acid at 100° and with boiling ethanol) at the end of the experiment was 18 and 10 % of the total dose when lungs weighing 27 and 15 g. respectively were perfused. About half of this radioactivity was in the tissue protein insoluble in 0·9 % sodium chloride solution.

Delayed appearance of label in secreted antibody

A consistent finding, both in these experiments and those with tissue slices (Askonas & Humphrey, 1958), was a definite delay between addition of [^{14}C]amino acid to the medium and its appearance in significant amounts in secreted antibody, although secretion of antibody into the medium proceeded linearly from the start. With perfused lungs this delay was half an hour or more and can hardly be attributed to time needed for the tissue to recover from the manipulations involved in setting up the perfusion, since the lung was perfused with complete medium for half an hour before labelled amino acids were added. Two possible explanations may be offered: antibody may be formed from some precursor (be it protein or bound amino acids) or it may be synthesized from an amino acid pool in equilibrium with the extracellular free amino acids, but may be bound in some way when first formed. The second is the explanation preferred by Peters (1957), who reported that in the liver albumin extracted from disrupted microsomes becomes labelled most rapidly and without appreciable lag period. Microsomal fractions isolated from spleen or bone marrow of immunized rabbits do contain some bound antibody extractable with carbonate buffer, pH 9·5, which disrupts the microsome structure (Askonas, 1958). It is therefore likely that antibody is first formed bound to subcellular particles, before it becomes freely extractable with isotonic medium. There is another lag period of about 30 min. between the appearance of radioactive free intracellular antibody and its appearance in the medium during incubation of sliced tissues (Askonas & Humphrey, 1958). The same is true *in vivo*. Whereas radioactive antibody can be extracted from tissues within 15 min. after injection of [^{14}C]amino acids, radioactive antibody appears in the blood stream only after 25 min. (Humphrey & Sulitzeanu, 1958). Hence it must take some time for the newly formed antibody to mix with the pool from which secretion occurs, or for the process of secretion, the transport of the protein molecules across the cell membranes, to take place.

Specific activity of free amino acids during perfusion

It is evident from Table 3 that at each stage of the perfusion the L-[^{14}C]valine of the medium was diluted with some inactive L-valine, presumably derived from the breakdown of lung protein (and possibly extracellular protein) to free amino acids. However, at the end of the experiment, the specific radioactivities of extracellular and intracellular valine were not identical, even though the tissue was immediately frozen in liquid air to prevent further changes. This suggests either that unlabelled free valine was continually being produced within the cells too fast for equilibrium to be reached with that outside, or that there must be some bound unlabelled intracellular valine not

readily exchangeable with the extracellular valine but easily extracted by the method used.

Since free valine refers to valine extractable with 10% trichloroacetic acid, it is possible that bound valine is released by the trichloroacetic acid and that a mixture of free and bound valine within the cells is determined. In each of three experiments, two with L-[^{14}C]valine and one with algal [^{14}C]-protein hydrolysate, the radioactivity of the intracellular amino acids was about 60% of that in the perfusion fluid. These findings are in accord with some observations reported in the literature. Loftfield & Harris (1956) administered 225 μmoles of L-[^{14}C]leucine to rats by repeated injections at short intervals of time, and found that intracellular leucine in the liver attained only 40% of the radioactivity of the leucine injected (the radioactivity of the blood leucine was not actually determined). Steiner & Anker (1956), using tissue cultures of spleen cells from an immunized donor in a medium containing a relatively large quantity of [^{14}C]-glycine, found that only about one-half of the glycine residues of the antibody were derived from added glycine after 48 hr. Considerations of this kind clearly must be borne in mind when attempts are made to calculate turnover rates of proteins by measuring incorporations of labelled amino acids.

Relative specific activities of intracellular antibody and free amino acids

It is evident from Table 3 that after perfusion for 5 hr. (1½ hr. after the last change of medium) the specific radioactivity of valine in the secreted antibody, and the antibody remaining in the lung cells, was only about one-sixth of that of intracellular free valine. Similar findings were obtained in two other experiments. If antibody was being synthesized from the intracellular free amino acid pool we examined, this finding implies a rather long turnover time. On the assumption that the mean specific activities of intracellular valine during successive perfusion periods of the experiment described in Table 3 were 800, 2500, 3100 and 3400 counts/min./100 μg. respectively; and from equation (3) derived by Humphrey & Sulitzeanu (1958), integrated into the form $y = x - ae^{-t/K}$, the turnover time K (time taken for cells to renew their antibody content) in this experiment can be calculated to be about 14 hr. This is at variance with our finding that the amount of antibody secreted in 5 hr. was approximately equal to that extractable from the lung at the end of an experiment, implying a turnover time of about 5 hr., and with the turnover time of 2–3 hr. deduced by Humphrey & Sulitzeanu (1958) for conditions *in vivo*.

Several possible explanations for the discrepancy may be suggested. The measured radioactivity of intracellular free amino acids may not represent those in the cells actually synthesizing antibody, and may be too high; there may be cells present which continue to secrete antibody, but which are no longer engaged in active synthesis, so that labelled antibody is thereby diluted with unlabelled; our methods of extraction may have led to serious (two- to three-fold) underestimation of the amounts of intracellular antibody present; or our theoretical assumptions may be erroneous. Until a homogeneous preparation of cells synthesizing specific antibody or other γ-globulin becomes available there appears to be no way of deciding between these possibilities. That the turnover time should prove longer in an isolated lung, perfused without blood, than it is *in vivo* is scarcely surprising. Nevertheless, it is possible that the estimate of 2–3 hr. quoted above, which involved numerous approximations, is too short.

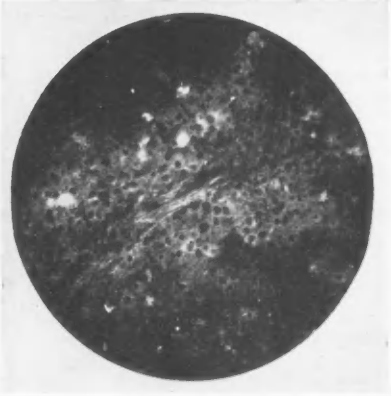
Mechanism of antibody secretion

If the intracellular antibody constitutes a pool from which secretion occurs into the medium, the specific radioactivities of newly secreted and of intracellular antibody at any time should be equal. It was not possible to measure instantaneously the radioactivity of antibody as it was secreted, and our experimental values for antibody in the medium represent average values for a perfusion period of 1–1½ hr., whereas those for intracellular antibody refer to antibody extracted at the end of the period. The two are therefore not strictly comparable, especially since equilibrium between the radioactive amino acids in the medium and in the cells was never completely attained. Nevertheless a comparison is of interest.

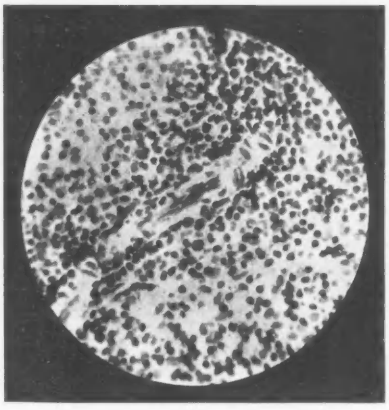
Table 2 summarizes six typical experiments. In three the specific radioactivity of intracellular antibody is almost equal to that of secreted antibody, and in three it is much lower. In our view, those experiments in which secreted and intracellular antibody had similar radioactivities at the end of the experiment support the idea that liberation of antibody into the medium can be described in terms of secretion from the total intracellular pool. In those experiments where the specific radioactivity of intracellular antibody was much less than that of secreted antibody, metabolically inert antibody must also have been present. There was nothing to suggest that this could be due to failure to perfuse part of the lung. However, a possible explanation may be that some cells contained stored antibody which was not continuously secreted.

The lungs in which the specific radioactivity of intracellular antibody was unexpectedly low all came from rabbits whose serum-antibody levels had begun to fall, despite continued injections of antigen. A section of the lung from such a rabbit,

stained to demonstrate antibody by the sandwich technique of Coons, Leduc & Connolly (1955) is shown in Fig. 4. In this lung there were many cells containing antibody in discrete granules, as well as cells with antibody uniformly distributed throughout the cytoplasm, and it seems reasonable to suppose that the antibody in granular form was metabolically distinct and relatively inert. We have not excluded the possibility that a similar condition may obtain to some extent in the lungs of all hyperimmunized rabbits, but in those which have not been overimmunized the granular form of antibody is comparatively unimportant.



(*a*)



(*b*)

Fig. 4. Section (×360) of lung from hyperimmunized rabbit whose plasma antibody level was falling, stained (*a*) by fluorescent antibody to show antibody-containing cells, and (*b*) the same field stained with haematoxylin and eosin. Note the varied appearance of antibody within the cells surrounding the arteriole.

Breakdown of globulin by immune lungs

The fact that hyperimmunized lungs were able to split iodide from ^{131}I-labelled γ-globulin is of some interest, since it is uncertain where plasma-protein breakdown occurs. Such lungs contain large numbers of macrophages, and these may be the cells responsible; if this is so, normal lungs would be less active, since their macrophage population is smaller. However, the maximum rate of breakdown which could be inferred in the experiment illustrated was 84 mg. in 24 hr., when the lung was perfused with serum diluted 1:10 in the medium. The level of total γ-globulin in the serum of this rabbit was about 50 mg./ml. If we assume that 10% of the 12 g. of total extracellular γ-globulin is broken down daily, the contribution to overall catabolism by the lung would be only 7%, unless catabolism were more rapid in the presence of higher concentrations of plasma protein. Gordon (1957) estimates that in the rat the liver is responsible for not more than 10% of plasma-albumin catabolism.

SUMMARY

1. Isolated lungs from hyperimmune rabbits, perfused *in vitro* with a balanced-salt solution containing amino acids and 10% of normal serum, secreted antibody into the medium at rates up to 8 mg./hr.

Allowance was made for extracellular antibody by injecting ^{131}I-labelled antibody into the rabbits beforehand, so that at the time of death extracellular antibody could be identified by its content of ^{131}I.

2. ^{14}C-Labelled amino acids added to the medium are incorporated into both specific antibody and other γ-globulin. The rate of synthesis of other γ-globulin was calculated to be rather greater than that of specific antibody.

3. A delay of more than 30 min. was observed between the addition of labelled amino acids and the appearance of labelled antibody in the medium. Antibody was extracted from the lung at the end of the experiments, and its radioactivity was compared with that of antibody secreted into the medium during the period immediately preceding. In some experiments the radioactivity of intracellular antibody was equal to, and in others it was less than, that of secreted antibody.

4. It is suggested that newly formed antibody mixes with an intracellular pool, from which secretion occurs into the medium. If this is so, antibody must first be synthesized in a bound form, and some lungs must contain antibody which is relatively inert metabolically.

5. The specific radioactivity of [^{14}C]amino acid in the perfusion fluid was measured at various times. The specific radioactivity of the intracellular free amino acids (L-valine or total amino acids), after perfusing for 5 hr., was about 60% of that of the corresponding amino acids in the medium at that time. The specific activity of the amino acids in the intracellular antibody was only one-sixth of that of the free amino acids on the intracellular pool. The interpretation of these findings is discussed.

6. Lungs from hyperimmune rabbits can liberate diffusible iodide from ^{131}I-labelled globulin.

We wish to thank Mr W. E. Brocklehurst for advice in the design of the perfusion apparatus, Mr R. C. Holloway for performing the iodinations and Mr G. Dickinson and Mr G. Savage for the gas counting.

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Transphosphorylations Catalysed by Bivalent Metal Ions

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In all enzyme reactions involving the breakdown or the formation of the polyphosphate chain of nucleoside triphosphates there is a requirement for bivalent metal ions. This requirement is absolute in most, if not all, cases. It is most commonly met by Mg^{2+} ions, but these can frequently be replaced by Mn^{2+} ions. A few enzyme reactions which show a requirement for a bivalent metal ion also show an additional requirement for a univalent metal ion. In the examples which have come to light so far, this additional requirement is met by potassium (Hers, 1952; Parks, Ben-Gershom & Lardy, 1957; Webster & Varner, 1954*a*). Of several reviews the one by Lardy (1951) is the most relevant to the present paper. It also gives references to earlier reviews.

The work presented here arose from a study of enzyme-catalysed exchange reactions. In the course of an experiment designed to test for an enzyme-catalysed exchange between radioactive orthophosphate and adenosine triphosphate, the disappearance of radioactive orthophosphate was observed in controls which contained no enzyme.

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The radioactive compound formed from orthophosphate was identified as pyrophosphate (Lowenstein, 1957*a*). The reaction was shown to depend on the presence of bivalent metal ions and adenosine triphosphate (ATP), and was formulated to proceed according to equation (1) (ADP represents adenosine diphosphate).

$$\text{ATP} + [^{32}\text{P}]\text{orthophosphate} \xrightarrow{Mn^{2+}} \text{ADP} + [^{32}\text{P}]\text{pyrophosphate} \quad (1)$$

This paper presents a detailed account and an extension of these observations.

A test of acceptors other than phosphates has shown that acylphosphate formation from nucleoside triphosphate and carboxylic acids also occurs in the absence of enzymes, and that it is catalysed by bivalent metal ions (Lowenstein, 1958).

EXPERIMENTAL

Materials and reagents

Nucleotides. The sodium salts of ATP and ADP were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. The free acid of adenosine monophosphate was obtained

from the Pabst Laboratories, Milwaukee, Wisc., U.S.A. The substances were dissolved in water, and adjusted to the required pH with KOH. The concentrations of the solutions were determined by measuring the light-absorption at 260 mμ, and the solutions were diluted to the required concentrations.

Radioactive phosphate. Carrier-free [^{32}P]orthophosphate was obtained from the Radiochemical Centre, Amersham, Bucks.

Analytical methods

'*Non-extractable phosphorus*'. The main analytical procedure was based on the method of orthophosphate determination of Berenblum & Chain (1938), and involves the extraction of acid phosphomolybdate with *iso*butanol. The reaction was stopped by the addition of 5 ml. of 1·5 N-H_2SO_4. The solution was transferred to a 20 ml. tube and 2·5 ml. of 5% (w/v) ammonium molybdate and 5 ml. of *iso*butanol were added. The tube was stoppered and shaken rapidly for about 30 sec. After the two layers had separated, the upper *iso*butanol layer was withdrawn by means of a capillary tube which led into a filter flask attached to a water pump. A further 5 ml. of *iso*butanol was added, and the extraction was repeated four times. The capillary suction tube was rinsed with 95% ethanol from a wash-bottle after each use. After the final extraction the aqueous layer was made up to 10 ml. with 95% ethanol, and the radioactivity of ^{32}P-labelled compounds remaining in the aqueous layer was measured in a liquid counter (Veall, 1948). Only orthophosphate is extracted into the *iso*butanol under these conditions, whereas phosphate esters and pyro- and poly-phosphates remain in the aqueous phase.

Orthophosphate. This was determined by the method of Berenblum & Chain (1938). The *iso*butanol extraction procedure was modified by using stoppered tubes instead of separating funnels. The unwanted aqueous layers were removed with the capillary suction tube already referred to above.

Pyrophosphate. Portions of ion-exchange fractions containing pyrophosphate were diluted to 7·0 ml. with N-H_2SO_4. The resulting solutions were heated in a boiling-water bath for 15 min. and cooled, and the orthophosphate liberated was determined.

RESULTS

Formation and identification of product of the reaction between adenosine triphosphate and orthophosphate. The incubation of ATP and [^{32}P]orthophosphate in the presence of Mn^{2+} ions and a buffer leads to the formation of a radioactive compound not extracted by *iso*butanol in the presence of acid molybdate. As is shown in Table 1, when ATP or the bivalent metal ions are omitted, the reaction is effectively abolished. When the buffer is omitted, the amount of product formed is reduced by an amount which is not easily controlled. This is presumably due to pH changes during the reaction in the absence of the buffer. As is shown below, the reaction has a sharp pH optimum.

The tentative identification of the reaction product by ion-exchange chromatography is shown in Fig. 1. A radioactive peak in the position of elution of pyrophosphate was observed in the chromatographic elution pattern after the reaction mixture had been incubated for 6 hr., but not at zero time. Moreover, the peak corresponding to the radioactive reaction product did not absorb light at 260 mμ and thus did not contain adenine. On the basis of this evidence the reaction product was presumed to consist of pyrophosphate. Radioactivity in the position of elution of ADP and ATP was negligible.

The identity of the radioactive reaction product was confirmed by adding authentic, unlabelled pyrophosphate to a portion of the reaction mixture and subjecting the whole to the chromatographic procedure shown in Fig. 1. The eluted fractions were assayed for total phosphorus and for radioactivity. The specific activities of the fractions representing the pyrophosphate peak were constant within the limits of the experimental error (Table 2). This establishes that the reaction product is pyrophosphate.

The specific activity of the pyrophosphate peak in the absence of added carrier pyrophosphate was found to be one-half to one-third that of the starting material.

Time course of the reaction. The influence of reaction time on the formation of pyrophosphate is shown in Fig. 2. The rate of formation of pyrophosphate is approximately constant during the first 8 hr. The slight decrease in the rate observed during this period and subsequently can be partially accounted for in terms of the pH, which dropped by about one unit in 24 hr.

Specificity of bivalent-metal-ion requirement. The effect on the transphosphorylation reaction of a number of different bivalent metal ions is shown in Table 3. An ATP/metal ion ratio of 1·11 was chosen in these experiments because some of the metal salts tested form insoluble precipitates with

Table 1. *Requirements for the formation of the reaction product*

The complete reaction mixture contained 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($1·16 \times 10^5$ counts/min.), 0·5 ml. of 0·10 M-ATP, 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer, pH 9·0, and 0·10 ml. of 0·45 M-$MnCl_2$. The final volume was 1·0 ml., temp. 37°, and time of incubation 5 hr.

Reaction mixture	Non-extractable radioactivity (μmole)
Complete	0·372
Buffer omitted*	0·232
ATP omitted	0·034
$MnCl_2$ omitted	0·012
Zero time	0·038

* pH adjusted to 9·0 with N-KOH.

ATP at slightly lower ratios. The results show that the effectiveness of Ca^{2+} and Cd^{2+} ions is of the same order of magnitude as that of Mn^{2+} ions, whereas the effectiveness of the other metals tested is about one order of magnitude less. Lead acetate was also tested under the conditions set out in Table 3, but no reliable results were obtained as to its effectiveness in the transphosphorylation reaction because the metal interfered with the analytical procedure. The effects of Ca^{2+} and Cd^{2+} ions were confirmed in a further experiment (Table 4). Lower concentrations of reactants were used in order to avoid the formation of precipitates

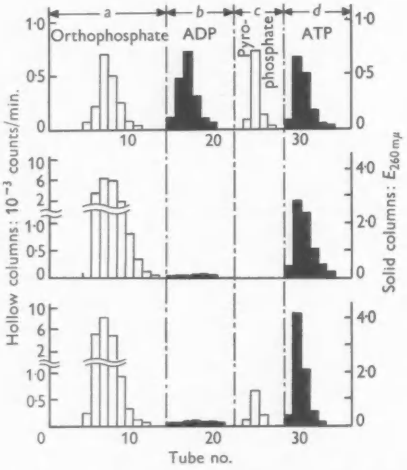

Fig. 1. Incorporation of radioactive orthophosphate into non-extractable compound. The reaction mixture contained 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($2·06 \times 10^5$ counts/min.), 0·50 ml. of 0·10 M-ATP, 0·10 ml. of 0·50 M-$MnCl_2$, 0·20 ml. of M-2-amino-2-hydroxymethylpropane-1:3-diol neutralized with HCl, pH 8·0 and 0·1 ml. of water; temperature 25°. Samples were withdrawn for analyses at 0 and 6 hr. One pair of samples was used for ion-exchange chromatography on columns of the formate salt of Dowex-2 resin, 10% cross-linked, 200–400 mesh, with a resin bed 2·5 cm. high and 1 cm. in diameter. The eluents employed are indicated by small letters: *a*, 0·15 M-ammonium formate; *b*, 3·0 N-formic acid; *c*, 0·50 M-ammonium formate; and *d*, M-ammonium formate. Fractions of 10 ml. were collected. The figure shows: top, elution pattern of a model mixture (for comparison); middle, reaction mixture at zero time; and bottom, reaction mixture after 6 hr. A second pair of samples was used for analyses of non-extractable radioactivity. The reaction was stopped by acidification with 5 ml. of 2·5 N-H_2SO_4, and orthophosphate was extracted as described under Analytical methods. The radioactivity not extracted by *iso*butanol rose from 60 counts/min./ml. at zero time to 9880 counts/min./ml. after 6 hr.

Table 2. *Identification of radioactive reaction product by co-chromatography*

The reaction mixture was similar to that described in Fig. 1. A sample of this solution, which contained about 1350 counts/min. of the reaction product, was co-chromatographed with approximately 4 μmoles of authentic pyrophosphate by the procedure described in Fig. 1. Radioactivity and phosphorus analyses for fractions 26–32 (eluent 0·5 M-ammonium formate) are shown below.

Fraction no.	Radioactivity (counts/min./fraction)	Phosphorus (μg./fraction)	Specific activity (counts/min./μg. of phosphorus) (±S.D.)
26	12·9	2·53	5·1±0·2
27	660	134·0	4·9±0·1
28	456	92·5	4·9±0·1
29	155	31·7	4·9±0·1
30	47·8	10·0	4·8±0·1
31	14·0	3·24	4·3±0·2
32	9·1	1·86	4·9±0·2

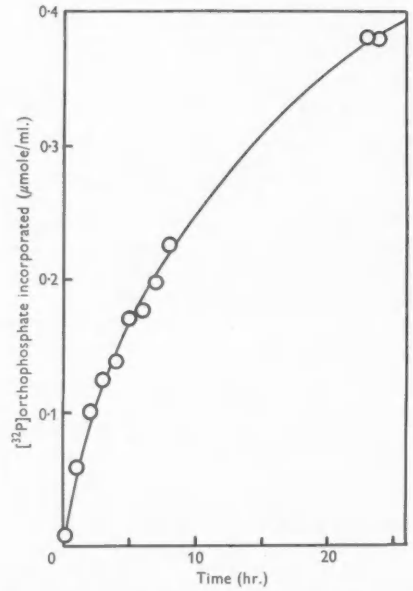

Fig. 2. Effect of time on transphosphorylation. The reaction mixture contained 2·0 ml. of 0·10 M-ATP (pH 10·0), 0·40 ml. of 0·50 M-$MnCl_2$, 0·40 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), 0·40 ml. of 0·05 M-[^{32}P]orthophosphate ($7·76 \times 10^4$ counts/min.), and water to a volume of 4·0 ml. The temp. was 30°. Samples of 0·20 ml. were withdrawn for analysis at the times indicated. pH at start, 9·0; pH after 24 hr., 8·0.

Table 3. *Activation of transphosphorylation by bivalent metal ions*

The complete reaction mixture contained 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($1{\cdot}16 \times 10^5$ counts/min.), 0·5 ml. of 0·10 M-ATP, 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and 0·10 ml. of 0·45 M-metal salt as indicated. The final volume was 1·0 ml., temp. 30°, and time of incubation 5 hr. Values of pyrophosphate formed are corrected for a blank containing no bivalent metal ions (0·012 μmole).

Metal salt	Pyrophosphate formed (μmole)
$MnCl_2$	0·360
$MnSO_4$	0·396
$BeSO_4$	0·022
$MgCl_2$	0·020
$CaCl_2$*	0·972
$ZnSO_4$	0·086
$CdCl_2$	0·244
$FeSO_4$	0·046
$CoCl_2$	0·062
$NiCl_2$	0·024
$CuSO_4$	0·030

* A precipitate formed in this tube.

Table 4. *Activation of transphosphorylation by bivalent metal ions*

The complete reaction mixture contained 0·05 ml. of 0·05 M-[^{32}P]orthophosphate ($8{\cdot}0 \times 10^4$ counts/min.), 0·25 ml. 0·10 M-ATP, 0·10 ml. 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and 0·05 ml. of 0·45 M-metal salt as indicated. The final volume was 1·0 ml. and temp. 30°.

Metal salt	Pyrophosphate formed (μm-moles) Zero time	After 5 hr.
None	—	0
$MnCl_2$	0	27
$CdCl_2$	0	47
$CaCl_2$	0	294

in the case of tubes containing Ca^{2+} ions. Zero-time controls were run as an additional check on the analytical procedure. A comparison of Tables 3 and 4 shows that the relative effectiveness of Mn^{2+}, Cd^{2+} and Ca^{2+} ions varies with the reaction conditions.

In these experiments no account has been taken of the possibility of a hydrolysis of pyrophosphate catalysed by metal ions.

pH *dependence of the* Mn^{2+}*-catalysed reaction.* The influence of pH on the formation of pyrophosphate is shown in Fig. 3. The transphosphorylation shows a sharp pH optimum, the reaction rates at pH 8·5 and 9·5 being only about one-half of the rate at pH 9·0.

Effect of the concentrations of Mn^{2+} *ions and adenosine triphosphate on transphosphorylation.*

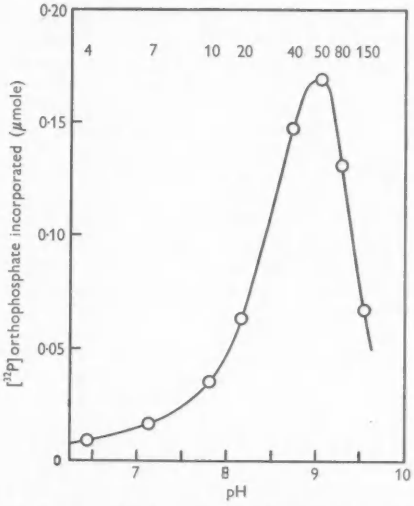

Fig. 3. Effect of pH on transphosphorylation. The complete reaction mixture contained 0·5 ml. of 0·10 M-ATP (pH 7·0), 0·1 ml. of 0·45 M-$MnCl_2$, 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($5{\cdot}0 \times 10^4$ counts/min.), and 2-amino-2-hydroxymethylpropane-1:3-diol (free base) to give the pH indicated. The pH of each sample was measured at the start of the reaction by a Beckman pH meter fitted with small electrodes. Figures above the experimental points of the curve indicate the amount of base added to the tube in question (in μmoles/ml.). The final volume was 1·0 ml., temp. 38°, and time of incubation 5 hr.

Fig. 4 demonstrates the effect of varying the concentration of Mn^{2+}. An optimum concentration of Mn^{2+} ions was not reached in the experiment, but the curve is beginning to flatten out at the highest concentrations of Mn^{2+} ions used. The maximum rate of transphosphorylation is therefore attained in a region where the ATP/metal ratio is less than 1. The amount of pyrophosphate formed decreases rapidly as the ATP/metal ratio is increased from 1·0 to 2·0, and it is virtually zero for ratios greater than 2·0. Moreover, if the central part of the curve is treated as an approximately straight line, and is produced to cut the abscissa, it does so at a point equivalent to an ATP/metal ratio of 2·0.

Fig. 5 demonstrates the effect of varying the concentration of ATP. An optimum ATP concentration is approached at an ATP/metal ratio of approximately 0·6. The ATP/metal ratio could not be decreased further under the conditions of the experiments shown in Figs. 4 and 5 because of the formation of precipitates. At higher ratios the reaction rate drops off, being 69 % of the optimum when the ratio is 1·0, and 9 % of the optimum when

the ratio is 2·0. If the right-hand part of the curve is treated as an approximately straight line and is produced to cut the abscissa, it does so at a point which is practically double the concentration of $MnCl_2$ used in the reaction. The transphosphorylation reaction therefore again practically ceases when the ATP/metal ion ratio is about 2·0.

Addition of ethylenediaminetetra-acetate to the reaction mixture reduces the rate of transphosphorylation to a level which is similar to that obtained at a metal-ion concentration equal to the difference between the total metal-ion concentration minus the ethylenediaminetetra-acetate concentration (Fig. 4).

Effect of orthophosphate concentration. The rate of transphosphorylation/μmole of orthophosphate is approximately constant over a limited range of orthophosphate concentrations. Under the conditions shown in Fig. 6 this range extends from 0 to 20 μmoles of orthophosphate/ml. Thereafter the rate/μmole of orthophosphate diminishes, at first presumably due to competition for the metal by orthophosphate, and subsequently because of precipitation of orthophosphate–metal complexes.

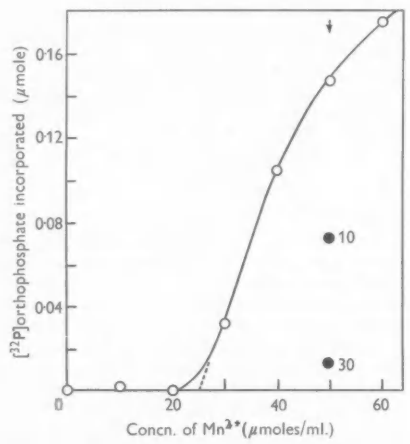

Fig. 4. Effect of concentration of Mn^{2+} ions on transphosphorylation. ○, Reaction mixtures which contained 0·5 ml. of 0·10 M-ATP (pH 9·0), 0·10 ml. of 0·05 M-[^{32}P]-orthophosphate ($3{\cdot}52 \times 10^4$ counts/min.), 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and $MnCl_2$ as indicated. ●, Reaction mixtures to which has been added ethylenediaminetetra-acetic acid, pH 9·0. The amounts added (in μmoles) are shown by the figures next to these points. The final volume was 1·0 ml., pH 9·0, temp. 36°, and time of incubation 5 hr. The arrow shows where the concentrations of ATP and $MnCl_2$ are equal.

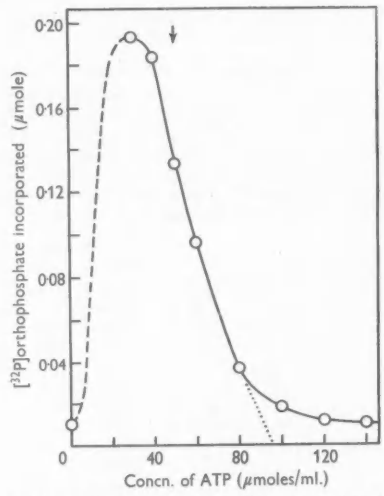

Fig. 5. Effect of concentration of ATP on transphosphorylation. The reaction mixture contained 0·10 ml. of 0·05 M-[^{32}P]orthophosphate ($1{\cdot}0 \times 10^5$ counts/min.), 0·1 ml. of 0·50 M-$MnCl_2$, 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and ATP as indicated. The final volume was 1·0 ml., pH 9·0, temp. 30°, and time of incubation 5 hr. Precipitation occurs at the ATP concentrations indicated by the broken part of the curve. The arrow shows where the concentrations of $MnCl_2$ and ATP are equal.

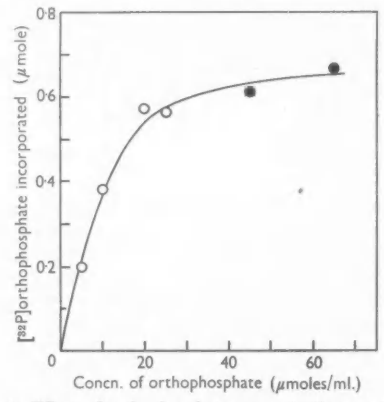

Fig. 6. Effect of orthophosphate concentration on transphosphorylation. The reaction mixture contained 0·5 ml. of 0·10 M-ATP (pH 9·0), 0·1 ml. of 0·50 M-$MnCl_2$, 0·10 ml. of 0·5 M-diethanolamine–diethanolamine hydrochloride buffer (pH 9·0), and [^{32}P]orthophosphate ($5{\cdot}26 \times 10^4$ counts/min.) at the concentrations indicated. The final volume was 1·0 ml., pH 9·0, temp. 30°, and time of incubation 5 hr. ○, Clear solution; ●, turbid solution.

DISCUSSION

The following abbreviations are used below. Nucleoside mono-, di- and tri-phosphate are represented by NP, NPP and NPPP respectively. Pyrophosphate is represented by PP. Radioactive phosphate is indicated by an asterisk, e.g. P*. Acceptor molecules are indicated by X. A chelate containing one molecule of NPPP and one metal ion will be designated ZM, a chelate containing one molecule of NPPP and two metal ions will be designated ZM_2, and a chelate containing two molecules of NPPP and one metal ion will be designated Z_2M.

The work reported here demonstrates the occurrence of a transphosphorylation reaction which is catalysed by bivalent metal ions, and which proceeds according to equation (1). The evidence for the reaction consists of the identification of pyrophosphate as the reaction product by isotope dilution (Table 1). The specific activity of the reaction product was also in accord with that expected from equation (1), and the formation of pyrophosphate was associated with an increase in the amount of ADP (Fig. 1).

The non-enzymic transphosphorylation reaction has a number of features in common with nucleoside triphosphate (NPPP) phosphotransferases. The most important of these is the bivalent-metal-ion requirement. Others include the inhibition of the reaction when the NPPP/metal ratio is greater than 1, the pH optimum (which falls within two units of that of most NPPP-phosphotransferases), the shape of the pH–activity curve and the temperature at which the reaction proceeds. A direct comparison of reaction rates is not possible since the rates of enzyme-catalysed reactions are by convention expressed in terms of enzyme concentrations. Moreover a comparison of rates with respect to the amounts of metal present in enzymic and non-enzymic transphosphorylations is not strictly justified in this case because the metal is not an integral part of the enzyme. However, one obtains the impression that the non-enzymic reaction is much slower than the enzyme-catalysed reaction.

The relative effectiveness of different bivalent-metal ions (Tables 3 and 4) varies with the reaction conditions and awaits detailed study. An interpretation of the pH-optimum curve (Fig. 3) probably involves many factors. Those which may have to be taken into account include the stability of ATP and pyrophosphate chelates at different pH values, the competitive binding of Mn^{2+} by hydroxyl ions at high pH values, pK_3 for orthophosphate and pK_4 for pyrophosphate. pK_4 for ATP can probably be ignored above pH 8·0.

The influence of the ATP/metal ratio on the rate of transphosphorylation is shown in Figs. 4 and 5. The rate increases as the ATP/metal ratio is decreased from 2·0 to 0·6. On the other hand, the rate of transphosphorylation tends towards zero as the ATP/metal ratio approaches 2·0, and it does not increase again at ratios greater than 2. These phenomena can be accounted for in terms of the existence of chelates with different ATP/metal ratios and with different net charges. A number of possible chelates and their interrelationships are shown in Fig. 7. Each of the individual chelates represented in Fig. 7 may occur in more than one structural form. For example, three possible structures of ZM^{2-} are shown in Fig. 8. Since pK_4 of ATP lies between 6·5 and 7·0 (Martell & Schwarzenbach, 1956; Smith & Alberty, 1956) the only species which need be considered at pH 9·0 are those in the lower line of Fig. 7, that is those derived from Z^{4-}. The experimental evidence shows that the chelates reactive in the transphosphorylation are either ZM^{2-} or ZM_2, or both, and that the species Z_2M^{4-} is not reactive. The decrease in the reaction rate as the ATP/metal ratio is increased from 1·0 to 2·0 is then most simply accounted for by the transition from mainly ZM^{2-}, at a ratio of 1·0, to mainly Z_2M^{4-} at a ratio of 2·0. The attainment of the maximum rate of transphosphorylation at ATP/metal ratios of less than 1·0 indicates that ZM_2 is the more reactive species.

The results also constitute evidence for the formation of the chelates Z_2M^{4-} and ZM_2. Stability constants for ZM have been measured by Smith & Alberty (1956), and by Martell & Schwarzenbach (1956), but none are available for ZM_2 and Z_2M. Martell & Schwarzenbach (1956) advance arguments that the stability constant for ZM_2 can be neglected by comparison with that for ZM in ATP–Mg and –Ca chelates. However, for manganese, Cohn (1954) mentions complexes with more than one metal atom/molecule of ATP. Although the ATP/metal ratio of the reactive chelate is thus not certain, for the following argument it is assumed to be 1·0. In such a chelate some of the negative charges of the polyphosphate chain are screened by the metal (Fig. 8). This screening greatly increases the probability of the attack on NPPP by anions which would otherwise

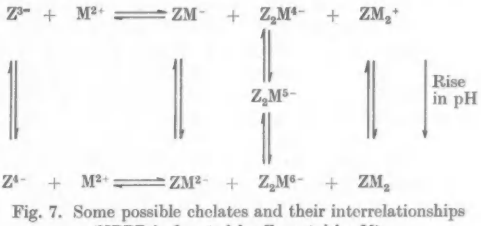

Fig. 7. Some possible chelates and their interrelationships (NPPP is denoted by Z, metal by M).

tend to be repelled, such as phosphomonoesters and carboxylic acids. For a transfer of the γ-phosphate from NPPP to an acceptor (equation 2), the chelate is probably that shown in Fig. 8*a* or *c*. In the former the metal is shown to span the β- and γ-phosphates of NPPP, and an attack on the γ-phosphate of the chelate by, for example, a phosphomonoester would take the course shown in Fig. 9*a*. The enzyme reactions may be formulated to proceed by a similar mechanism (Fig. 9*b*) except that at pH 7·4 the reactive chelate may be derived from either Z^{4-} or Z^{3-}. An attack on the α- or β-phosphates of NPPP, which occurs in enzyme reactions of the type shown in equations (3) and (4), could proceed by a similar mechanism.

$$\text{NPPP} + \text{X} \rightarrow \begin{cases} \text{XP} + \text{NPP} & (2) \\ \text{NPX} + \text{PP} & (3) \\ \text{XPP} + \text{NP} & (4) \end{cases}$$

If the reactive chelate in reactions (2), (3) and (4) is that shown in Fig. 8*c*, the enzyme may exert its specificity by influencing the charge distribution along the polyphosphate chain. On the other hand, the chelate involved in reaction (3) may be that shown in Fig. 8*b*, and that involved in reaction 4 may be either that shown in Fig. 8*a* or that shown in Fig. 8*b*.

Certain enzymic transphosphorylations from NPPP result in the phosphorylation not of anions but of neutral molecules. Such reactions can be formulated as nucleophilic attacks by the neutral molecules on a phosphorus atom of NPPP. In these cases chelation also greatly increases the electrophilic character of the phosphorus atom, and thus facilitates the attack by the neutral molecule.

It has been proposed that the mechanism of action of ATP-phosphotransferases involves the phosphorylation of the enzyme by ATP. Further, the formation of a phosphorylated enzyme was postulated to represent one general mechanism for the utilization of ATP in enzyme reactions (Webster & Varner, 1954*a*, *b*; Snoke, 1953; Jones, Lipmann, Hilz & Lynen, 1953; Lipmann, 1954). If reactions (2), (3) and (4) proceed by a mechanism involving the formation of a phosphorylated enzyme as an intermediate step, the enzymes concerned should catalyse exchange reactions between NP*P* and NPPP, P*P* and NPPP, and NP* and NPPP respectively, and the exchange should occur in the absence of acceptor X. This should be so unless it is postulated that the acceptor must be present to fulfil some purpose other than acting as acceptor. On the other hand, in the enzymic mechanism of transphosphorylation shown in Fig. 9*b* the enzyme does not act as a phosphoryl carrier, and no exchange should be observed in the absence of acceptor X. Exchange criteria of this type have been employed by Berg (1956) for the acetate-activating enzyme, and have led him to propose

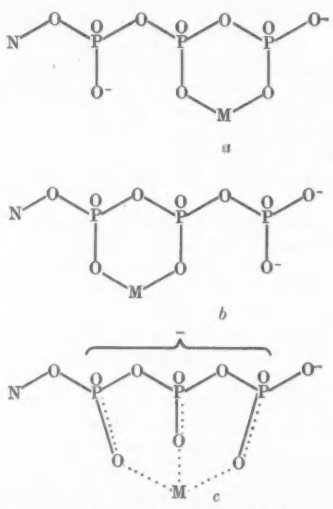

Fig. 8. Three possible structures of the chelate ZM^{2-}. In *a* the metal spans the α- and β-phosphates, in *b* the β- and γ-phosphates, and in *c* all three phosphates. In *c* one of the charges may be on any one of the three phosphates.

enzymic mechanisms for the formation of acetyl phosphate and adenyl acetate similar to those shown in Fig. 9. A similar mechanism is probably also operative in the non-enzymic activation of acetate demonstrated by Lowenstein (1958). Chemical mechanisms in enzymic transfer reactions have also been discussed by Koshland (1954) and by Boyer & Harrison (1954).

The non-enzymic reaction and the results of enzymic-exchange experiments (Berg, 1956; Lowenstein, unpublished observations) show that transphosphorylations from ATP need not proceed via phosphorylated enzymes. This conclusion does not apply to reactions which do not require bivalent metal ions or which involve the transfer of phosphate groups with a free energy of hydrolysis comparable with that of the common sugar phosphates. A case in point is phosphoglucomutase, which has been shown to be phosphorylated in the course of the reaction that it catalyses (Anderson & Jollès, 1957; Kennedy & Koshland, 1957).

Metal chelates of NPPP have been postulated to be the 'true' substrates in enzyme reactions involving nucleotides, the role of the metal being to bind the nucleotide to the enzyme (for a discussion see Calvin, 1954). The reaction demonstrated above shows that the role of the metal ion in enzymic transphosphorylations goes beyond that of a passive binding function. Instead the enzyme may be considered to make more specific the non-enzymic mechanism by the selective adsorption of

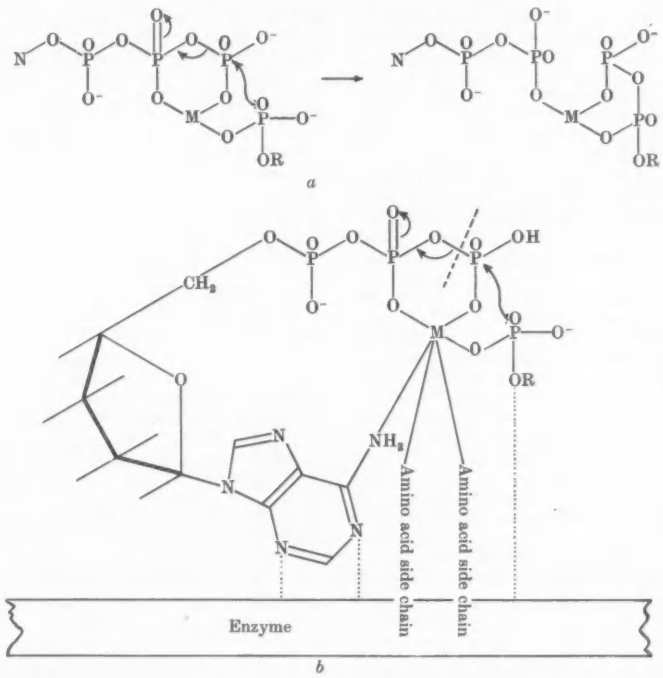

Fig. 9. *a*, Mechanism of non-enzymic transphosphorylation by the nucleophilic attack of a phosphomonoester on the γ-phosphate of NPPP. *b*, Diagrammatic representation of the same mechanism in enzymic transphosphorylation (only the initial attack is shown).

specific nucleotides and specific acceptors. It may do this by binding the NPPP–metal chelate to the enzyme surface through vacant co-ordination valencies of the metal. In addition the chelate may complex with the acceptor molecule as is suggested in Fig. 9*b*. No experimental data exist on the binding of NPPP–metal chelates to enzymes, but it has been demonstrated by Murphy & Martell (1957) that the co-ordination of metals with simple peptide linkages is too weak to account for the strength of co-ordination which has been observed with ions as basic as Mg^{2+} and Mn^{2+}. Thus binding of these metals must occur through groups, such as carboxyls and iminazoles, available on the side chains of amino acids.

The substrate concentrations commonly employed in enzymic transphosphorylations are approximately one-fiftieth of those employed in the non-enzymic reaction. These differences can be partially reconciled in that the adsorption and orientation of the substrates on the enzyme surface is, other influences apart, tantamount to a localized raising of the substrate concentrations.

Polyphosphates in the form of partially hydrated polyphosphoric acid and partially hydrated phosphoryl chloride have been employed as phosphorylating agents (for references see Seegmiller & Horecker, 1951; Lowenstein, 1957*b*). The compounds phosphorylated were generally alcohols, the reaction conditions usually involved heating, and no catalysis by bivalent metal ion was reported. The reaction conditions were thus quite different from those reported here.

SUMMARY

1. A non-enzymic transphosphorylation has been demonstrated to occur between adenosine triphosphate and orthophosphate.

2. The reaction is catalysed by bivalent metal ions. Of those tested, Mn^{2+}, Ca^{2+} and Cd^{2+} were found to be the most effective.

3. The reaction has a sharp pH optimum. With Mn^{2+} ions the rate of transphosphorylation is optimum at adenosine triphosphate/metal ratios between 0·6 and 1·0.

4. A mechanism is proposed for the reaction, and its relation to enzymic transphosphorylation is discussed.

I am indebted to Mr Roy Cooke for invaluable technical assistance. My thanks go to Professor Sir Hans A. Krebs, F.R.S., and to Drs W. Bartley, K. Burton and R. J. Williams for reading the manuscript.

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The Biosynthetic Preparation of [16-^{3}H]Aldosterone and [16-^{3}H]Corticosterone

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For both metabolic *in vivo* and analytical *in vitro* uses of radioactive steroids it is an advantage to add amounts of the labelled compound much smaller than are already present in the animal or extract but sufficiently radioactive to be followed with ease through any process. [4-^{14}C]Cortisol of sufficiently high specific activity (about 0·004 μc/μg.) for most purposes is now available. However, [4-^{14}C]corticosterone of similar specific activity is not suitable for many applications owing to the low rate of secretion of this hormone in man. (Ayres *et al.* 1957*a*; Peterson, 1957). Radioactive aldosterone, of specific activity high enough to be useful in metabolic studies, has not previously been prepared.

The advantages in the preparation and use of tritiated steroids are the ease and cheapness of their synthesis at high specific activities and their relative radiation safety for metabolic studies in man. The disadvantages of their use are the difficulty of assay, which is overcome by the application of methods employing a flow counter (Banks, Crawhall & Smyth, 1956) or a liquid scintillator (Davidson & Feigelson, 1957), and also the possibility of unstable labelling. This latter consideration has been discussed elsewhere (Pearlman, 1957*b*) and found to be unlikely for most metabolic applications of [16-^{3}H]progesterone. The same remarks apply to the use of 16-^{3}H-labelled corticosteroids.

The preparation of [16-^{3}H]progesterone of high specific activity has been described previously (Pearlman, 1957*a*). Progesterone has been shown to be an intermediate in the biosynthesis of aldosterone and corticosterone by capsule strippings of

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ox adrenal gland (Ayres, Hechter, Saba, Simpson & Tait, 1956*b*). This tissue has been shown to consist mainly of zona glomerulosa which, on incubation, produces much more aldosterone per gram of tissue than does whole adrenal cortex (Ayres, Gould, Simpson & Tait, 1956*a*). [16-^{3}H]Aldosterone and [16-^{3}H]corticosterone of high specific activity (about 2 μc/μg.) can therefore be prepared by incubating [16-^{3}H]progesterone with such tissue. A preliminary account of some of this work has been published (Ayres *et al.* 1957*a*).

MATERIALS AND METHODS

General

Solvents, kieselguhr (Celite 545) and silica gel for column chromatography and general use were prepared as previously described (Ayres, Garrod, Simpson & Tait, 1957*b*). For crystallization, ethanol was purified by refluxing over magnesium ethoxide, followed by distillation through a short column; benzene and light petroleum were purified by shaking with sulphuric acid, followed by washing with water and a 10% (w/v) aqueous solution of sodium carbonate. They were then dried over anhydrous calcium chloride and distilled through a short column. The light petroleum was of boiling point 80–100° except where stated otherwise.

Samples of authentic crystalline corticosterone and cortisol for melting-point comparisons were kindly given by Glaxo Laboratories Ltd., and the Medical Research Council Steroid Reference Collection respectively.

Melting points were determined on a Kofler block and are corrected.

Duralumin planchets for the assay of tritium were obtained from A. and H. Hall, 67 Elmers End Road, Beckenham, Kent, and were thoroughly scrubbed and washed in chloroform before use.

The Perkin–Elmer double-beam instrument, Model 21, was used for infrared-absorption spectroscopy. This was carried out in carbon disulphide solution (3 mm. microcell) for aldosterone diacetate and corticosterone monoacetate, in chloroform solution (1 mm. microcell) for cortisol monoacetate, aldosterone diacetate and corticosterone monoacetate and in a potassium bromide disk for cortisol monoacetate. Ultraviolet-absorption spectroscopy was carried out in ethanol (0·5 cm. cells) in the Hilger Uvispek H 700.

Acetylation was carried out by dissolving the steroid in 0·3 ml. of pyridine and adding 0·15 ml. of acetic anhydride. The resulting solution was left overnight at room temperature and then evaporated to dryness at 20°.

Assay of tritium

By flow-counter. The tritiated material was deposited from 0·2 ml. of a chloroform solution to cover completely a circular planchet, 2·5 cm. in diameter, which had a large number of circular grooves, about 0·05 mm. deep, scored on its surface. This ensured fairly uniform distribution of the material. The planchet was dried at 45° and assayed in a flow counter (Tracerlab Inc., Boston 10, Mass., U.S.A., Model SC18).

In preliminary experiments it was found that, if various weights of progesterone of equal total radioactivity were assayed in this way, the counting rate was greatly depressed from 0 to 60 μg. and then decreased more gradually for additional quantities. Fig. 1 shows the results obtained for different amounts of progesterone added to a constant quantity of [16-^{3}H]progesterone. Presumably the initial rapid drop in the count rate is due to the absorption of back-scattered β-rays of very low energy. The half-value thickness, calculated from the second part of the curve (approx. 60 μg./cm.^{2} solid) corresponds to what would be expected from the energy of the primary β-rays of tritium.

Ten samples were counted for each particular weight of material and the standard deviation calculated (Fig. 1). The coefficient of variation is about 12% for samples of negligible weight but decreases to 6% for weights above 100 μg. This large variation at negligible weight may be due to very small but variable amounts of grease which are impossible to remove by ordinary cleansing processes. This material would have a considerable effect on the count rate on the initial part of the curve of count rate against weight ($<$40 μg. of solid), but much less on the second part of the curve. It is therefore necessary, for accurate work, to add at least 100 μg. of progesterone to the specimen and we have used 160 μg. of the steroid for this purpose. When this is done, the extra addition of 10 μg. of all substances so far tested, including corticosterone, cortisol, progesterone, deoxycorticosterone and beeswax, has no significant effect on the counting rate.

Different amounts of wax added to a negligible but constant weight of [16-^{3}H]progesterone give rise to a similar curve to that shown in Fig. 1, but the extra addition of 10 μg. of various steroids to 160 μg. of wax significantly lowers the counting rate below that obtained by adding 10 μg. of wax. When stearic acid is used as the major component, the same effect can be demonstrated.

As a routine procedure, therefore, if the sample weighs less than 10 μg., it is dissolved in 0·3 ml. of a solution of progesterone (800 μg./ml.) in chloroform and 0·2 ml. of the mixture placed on the planchet. The counting efficiency of this procedure is 16% and the coefficient of variation 6·5%, if random statistical counting errors are negligible.

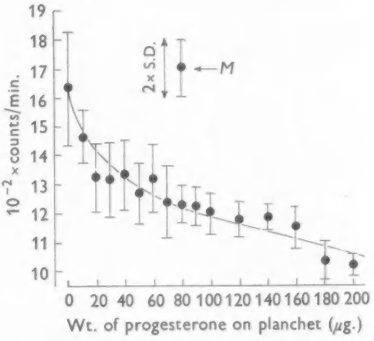

Fig. 1. Counting rate plotted against weight of progesterone on planchet. A negligible but constant weight of [16-^{3}H]progesterone was added to all planchets. The mean (M) and standard deviation at a particular weight were obtained by counting ten samples.

If the sample is of greater weight, it is made up to at least 160 μg. with progesterone and counted on the planchet. An equal quantity of this mixture plus a negligible weight of radioactive [16-^{3}H]progesterone standard, giving a high counting rate at least five times that of the sample, is also counted, from which a correction for self-absorption can be applied to the original count. The coefficient of variation of this method, which has successfully been applied to the assay of various crude extracts, is about 9% if random statistical counting errors are negligible. To avoid possible charging effects (Banks *et al.* 1956) samples less than 2 mg. (<300 μg./cm.^{2}) were used.

It was also possible to assay after paper chromatography and development of the soda fluorescence of the tritiated steroids. The appropriate area of the developed paper was counted in the flow counter with an overall efficiency of 0·24% for Whatman No. 2 paper before spraying with sodium hydroxide solution and 0·22% after spraying and heating.

By bremsstrahlung *emission.* It was convenient, when dealing with large amounts of tritium, to assay samples by their *bremsstrahlung* emission. The planchet, prepared as described above, was counted by a G.E.C. type E.H.M. 2 mica-window counter giving 1·3 counts/min./μc of tritium. Other sources, such as the steroid deposited on a glass slide, gave similar counting efficiences. No gain in efficiency was obtained by using a Mullard MX 118 soft X-ray Geiger tube. The half-value absorption thickness for the *bremsstrahlung* radiation was about 6 mg./cm.^{2} of cellophan, and hence self-absorption effects were usually negligible.

Standardization. A combustion method (Avivi, Simpson, Tait & Whitehead, 1954) was used to obtain the overall efficiency of the counting procedures and also the absolute activity of the steroids. The activity of a sample of [16-^{3}H]progesterone was thus compared with tritiated water, supplied by A.E.R.E., Harwell, and calibrated there by mass spectrometry. Therefore all values given in this paper for efficiencies and activities are in terms of this standard water sample.

EXPERIMENTAL

Preparation of [16-^{3}H]*progesterone*

3β-Hydroxypregna-5:16-dien-20-one acetate (589·5 mg.) was hydrogenated with 20 c of tritium gas (7·9 ml. at s.t.p., 98·7% of H) as described by Pearlman (1957*a*). Partition chromatography instead of chromatography on alumina was used for isolating [16-^{3}H]progesterone from the products of the Oppenauer oxidation stage. The solvent system methanol–water (4:1, v/v)/light petroleum was used with 80 g. of Celite 545 and 40 ml. of stationary phase in a 3 cm. diameter column, $R_{\text{progesterone}}$ 0·25. Crystalline [16-^{3}H]progesterone (107 mg.), m.p. 121°, was obtained from light petroleum, $[\alpha]_D^{15}$ +190 $\pm$9° in chloroform (*c*, 0·1), light-absorption maximum 240 mμ (ϵ 16 300). The specific activity was 6·35 μc/μg.

Incubation of progesterone, extraction and preliminary purification of adrenocortical steroids

This preparation of [16-^{3}H]progesterone (96·5 mg.) was incubated with a total weight of 3·3 kg. of capsule strippings of ox adrenal gland.

Ox adrenal glands were obtained from the slaughter-house approximately 40 min. after death and then placed at 0° until reaching the laboratory. The glands were then cleaned of all fat and connective tissue except the capsule. A slit was made along one side of the gland by passing a scalpel beneath the capsule, the blade being kept parallel with the capsule surface. The capsule was then peeled off with forceps. This tissue (8 g./flask) was incubated with 234 μg. of [16-^{3}H]progesterone and 80 ml. of Krebs–Ringer solution (Krebs, 1933) buffered at pH 7·4 with 0·04 M-2-amino-2-hydroxymethylpropane-1:3-diol. The solution also contained 3 mM-sodium fumarate, 3 mM-nicotinamide and 10 mM-glucose. Incubation was carried out in oxygen at 37° with shaking for 2 hr. The medium was then drained from the tissue and extracted three times with an equal volume of chloroform at room temperature, the emulsion being broken by centrifuging at 5°. The chloroform extract was then evaporated to dryness in an evaporator (Craig, 1950) and the solvent recovered with condensers maintained at -20°. The extract (from 72 g. of tissue at a time) was then dissolved in 100 ml. of ethyl acetate–light petroleum (1:1, v/v) and the solution put on a 2 cm. diameter column containing 15 g. of silica gel. The corticosteroids were eluted with 50 ml. of ethyl acetate–methanol (1:1, v/v), which was then evaporated to dryness (Bush & Sandberg, 1953). Of the added radioactivity 66% was found in this fraction. Less than 1% appeared in the ethyl acetate–light petroleum eluate or in methanol strippings of the column. The dried material from the ethyl acetate–methanol fraction was then extracted with ethanol at 0°. This removed over 99% of the radioactivity present and achieved considerable purification, particularly from material which tended to clog the subsequent partition columns.

Preliminary analysis

Values for the total corticosterone, aldosterone and cortisol were obtained in the following manner. A portion from the ethanol extract, equivalent to 17 g. of tissue, was chromatographed on several partition columns, each of which had a resolution of 1000 theoretical plates. The first column separated corticosterone ($R_{\text{corticosterone}}$ 0·9) from a mixture of cortisol and aldosterone (R 0·48), both of which ran at the same speed in this solvent system [methanol–water (1:1, v/v)/benzene–ethyl acetate (21:4, v/v), system *A*]. They were completely separated from one another on a second column system ($R_{\text{aldosterone}}$ 0·32, R_{cortisol} 0·18), with methanol–water (1:1, v/v)/benzene, system *B*. Aldosterone was further purified on another column ($R_{\text{aldosterone}}$ 0·10) with methanol–water (1:1, v/v)/benzene–light petroleum (7:3, v/v), system *C*. The corticosterone was also further purified on another column system ($R_{\text{corticosterone}}$ 0·32), with methanol–water (4:1, v/v)/benzene–light petroleum (17:3, v/v), system *D*. All columns were 60 cm. in length and 1 cm. in internal diameter and contained 24 g. of Celite 545 and 12 ml. of stationary phase.

Portions of the eluted fractions from the columns were chromatographed on paper in the Bush *C* system (Bush, 1952) for cortisol and aldosterone and a modified *B*2 system [methanol–water (4:1, v/v), stationary phase] for corticosterone. The amounts of steroid in the fractions of the final column of each particular compound were estimated by comparing their soda fluorescence with that of standards by a fluorimeter after paper chromatography (Ayres *et al.* 1957*b*). During the purification of the steroids

Table 1. *Amounts and specific activities of corticosterone, aldosterone and cortisol produced*

The amount produced is the total from 3·3 kg. of capsule strippings (corrected for recovery) and is according to preliminary analysis.

Compound produced	Amount produced (mg.)	Specific activities (μc/μg.) Column fractions of free compound before crystallization	Cryst. free compound	Column fractions of acetyl derivative
Corticosterone	59	2·77 ±0·1	$2{\cdot}6_2 \pm 0{\cdot}1$	$2{\cdot}7_7 \pm 0{\cdot}1$
Aldosterone	9·9	1·74 ±0·07	1·82±0·07	1·75±0·07
Cortisol	8·2	$0{\cdot}76_5 \pm 0{\cdot}03$	0·76±0·03	0·72±0·03

the recovery was kept as high as possible (all fraction tubes containing the appropriate steroid were pooled for the next column). From the extract of the incubating fluid to the final paper spot, the recovery was about 80% in all three cases. An estimate of aldosterone made by direct paper chromatography of the extract on the Bush *B*5 system [methanol–water (1:1, v/v)/benzene] was about 35% higher than that after purification, even after correction for recovery. This was due to the presence in the original extract of an unidentified compound giving soda fluorescence and moving only slightly slower than aldosterone in the Bush *B*5 system. This compound was completely separated from aldosterone by the same solvent system on a column (system *B* and also in system *C*). The incubation gave 2·5, 3·0 and 18 μg./g. of tissue for cortisol, aldosterone and corticosterone respectively, all values being corrected for recovery (Table 1).

Isolation of steroids

The ethanol extract from 3·3 kg. of tissue was purified on a partition column, 3 cm. in diameter and 60 cm. long, containing 80 g. of Celite 545 and 40 ml. of stationary phase. Solvent system *A* was used, which again separated corticosterone from a mixture of cortisol and aldosterone.

Corticosterone. The corticosterone was further purified on four columns by solvent system *D*. The first column contained 80 g. and the other three 24 g. of Celite 545, with corresponding column dimensions (60 cm. × 2 cm. or 1 cm. diameter) and volumes of stationary phase (always half the weight of Celite, v/w). The quantities of steroid in the fractions of the final column were estimated by u.v. absorption (taking ϵ 16 500 at u.v.-absorption maximum 240 mμ) and tetrazolium reaction in solution (Mader & Buck, 1952) compared with that of authentic corticosterone. The tritium was assayed by flow counter and from *bremsstrahlung* emission. For those fractions containing appreciable steroid (usually arranged to be a maximum of three, height of theoretical plate 0·7 cm. for these columns), the estimate by u.v. absorption and tetrazolium reaction agreed within the limits of experimental error. Paper chromatography in the modified Bush *B*2 system of a portion of these fractions revealed only one spot. This gave equivalent and symmetrical soda-fluorescence and tetrazolium reactions quantitatively in agreement with the measurements carried out in solution. The amount of steroid and radioactivity corresponded in all fractions and the mean specific activity from the two tubes containing most steroid was $2{\cdot}7_5 \pm 0{\cdot}1$ μc/μg. (Table 1).

These two fractions were pooled and a portion taken for crystallization, which was carried out from ethanol. The crystals were washed with ethanol at 0° and light petroleum (b.p. 40–60°) at room temperature and dried over silica gel *in vacuo* at 60° for 15 hr. Crystals (10·8 mg.) were obtained, m.p. 179–182·5°, u.v.-absorption maximum 240 mμ. Authentic corticosterone gave m.p. 180–182·5°; a mixed melting point with the radioactive crystals was 179–182°. A solution of the crystals gave equivalent u.v. absorption at 240 mμ and tetrazolium reaction and also soda-fluorescence and tetrazolium reaction after paper chromatography in the Bush *B*2 or Zaffaroni propylene glycol–toluene systems. Only one spot could be seen after both these reactions as a result of paper chromatography in the two systems, and this ran at the same speed as corticosterone. Over 97% of the radioactivity was coincident with the soda-fluorescence area. The specific activity of the crystalline material was $2{\cdot}6_2 \pm 0{\cdot}1$ μc/μg. (Table 1).

The washings from the crystallization were acetylated and a portion was chromatographed in the Bush *B*3 paper system. Only one symmetrical and coincident spot could be seen after soda-fluorescence and tetrazolium reaction and this ran at the same speed as corticosterone acetate. The remainder was chromatographed on a column containing 24 g. of Celite 545 and with solvent system methanol–water (4:1, v/v)/light petroleum–benzene (3:2, v/v), system *E* ($R_{\text{corticosterone}}$ 0·28). The radiochemical purity of the fractions containing corticosterone acetate was investigated in a similar manner to that described for the final column of the free compound and was satisfactory. The steroid in the peak fractions (containing 4·1 mg. of corticosterone acetate) was investigated by infrared-absorption spectroscopy; about 250 μg. gave spectra (including the carbonyl and fingerprint regions) identical with that of authentic corticosterone acetate. The mean specific activity of the two peak fractions was $2{\cdot}7 \pm 0{\cdot}1$ μc/μg. (Table 1). A portion of the crystalline free compound was also acetylated and gave one spot in the Bush *B*2 paper system. This had the same R_F as corticosterone acetate. Over 95% of the radioactivity was coincident with the soda-fluorescence area.

Aldosterone. The aldosterone was chromatographed on a second column with 80 g. of Celite 545 and solvent system *A* and then separated from cortisol and the other major impurity, previously mentioned in the description of the preliminary analysis, by two further 24 g. columns, with solvent system *B*. The final 24 g. column employed as a solvent system methanol–water (1:1, v/v)/benzene–ethyl acetate (19:1, v/v) ($R_{\text{aldosterone}}$ 0·40), system *F*. The purity of the steroid in the fractions of this column was examined as described for corticosterone except that the Bush *B*5 paper system was used. It was satisfactory according to the same criteria. The mean specific activity

from the two tubes containing most steroid was $1{\cdot}74\pm0{\cdot}07\,\mu c/\mu g$. (Table 1).

These two fractions were pooled and crystals obtained from 10% (v/v) aqueous ethanol. The crystals were washed with ethanol at $-5°$ and light petroleum (b.p. 40–60°) at room temperature, and dried over P_2O_5 *in vacuo* at 45° for 15 hr. This gave 1·87 mg. of crystals, m.p. 111–115°, re-melting at 156–162° with some small remnants up to 165°, u.v.-absorption maximum 240 mμ. We have been unable to obtain for comparison synthetic (+)-aldosterone crystals of high melting point. The final melting point of crystals from natural sources as determined by Kofler block has been reported as 153–158° (Simpson *et al.* 1953); 157–163° (Simpson & Tait, 1955, carried out under conditions identical with those of the present study); 156–165° (Luetscher, Dowdy, Harvey, Neher & Wettstein, 1955). A solution of crystalline material gave equivalent u.v. absorption ($\lambda_{max.}$ 240 mμ, ϵ 16 500) and tetrazolium reaction as compared with authentic aldosterone. The estimate obtained from these two methods was in agreement with that from soda-fluorescence and tetrazolium reactions after paper chromatography in the Bush *B*5 or *C*, the E_2B (Eberlain & Bongiovanni, 1955) or Zaffaroni propylene glycol–toluene systems. After both reactions only one spot could be seen in all four systems and this ran at the same speed as aldosterone. Over 95% of the radioactivity was coincident with the soda-fluorescence area. The specific activity of the crystalline material was $1{\cdot}8_2\pm0{\cdot}07\,\mu c/\mu g$. (Table 1).

The washings from the crystallization were acetylated and a portion run in the Bush *B*3 paper system. After soda-fluorescence and tetrazolium development only one spot could be seen. This had the same R_F as had aldosterone diacetate. The remaining steroid was chromatographed on a 24 g. column, with solvent system *E* ($R_{\text{aldosterone diacetate}}$ 0·35). The radiochemical purity of the fractions containing aldosterone diacetate (total 900 μg.) was satisfactory by the usual criteria. About 250 μg. gave an absorption spectrum in the infrared, including the carbonyl and fingerprint region, identical with aldosterone diacetate. The mean specific activity of the two fractions containing most steroid was $1{\cdot}7\pm0{\cdot}07\,\mu c/\mu g$. (Table 1).

Some of the crystalline compound was acetylated and gave one spot running at the same speed as corticosterone acetate ran in the Bush *B*3 system. Over 97% of the radioactivity was coincident with the soda-fluorescent area. A portion of the acetylated material was mixed with aldosterone [*carboxy*-^{14}C]diacetate prepared from authentic aldosterone (Ayres *et al.* 1957*b*) and chromatographed on a column, with solvent system *E*. The tritium was assayed by flow counter (the counts being corrected for the small contribution from the ^{14}C β-rays) and the ^{14}C on glass slides by a mica-window counter (Ayres *et al.* 1957*b*). The ^{3}H/^{14}C ratio was the same in all fractions within the limits of experimental error and $99\pm5\%$ of the counts originally present as free [16-^{3}H]aldosterone appeared in the fractions containing ^{14}C. This figure corresponds to the product of the percentage purity of the original aldosterone multiplied by the percentage yield to diacetate on acetylation.

Cortisol. The cortisol was further chromatographed on a second 80 g. column, with solvent system *A* and another three 24 g. columns employing systems *B*, *F* and *A*, in that order. The radiochemical purity of the free compound, in the fractions of the final column and after crystallization, and that of the cortisol monoacetate, in the fractions of its column, were investigated as described for aldosterone and corticosterone and found to be satisfactory. The relevant specific activities obtained were $0{\cdot}76_8\pm0{\cdot}03$, $0{\cdot}76\pm0{\cdot}03$ and $0{\cdot}72\pm0{\cdot}03\,\mu c/\mu g$. respectively (Table 1). The paper-chromatographic systems for investigating the free compound were as for aldosterone. The modified Bush *B*2 paper system and a 24 g. column, with solvent system *D*, ($R_{\text{cortisol monoacetate}}$ 0·45) were used to isolate and investigate the acetyl derivative.

Crystals of [16-^{3}H]cortisol were obtained from 50% aqueous ethanol and were washed with ethanol at 0° and light petroleum (b.p. 40–60°) at room temperature, then dried over silica gel *in vacuo* at 60° for 30 hr. Crystals (2·29 mg.) were obtained, which gave m.p. 217–220°, $\lambda_{max.}$ 241 mμ. Authentic cortisol gave m.p. 216–219°, mixed m.p. 215–219°. A further 950 μg. of cortisol monoacetate was obtained by acetylating the washings from the crystallization.

No attempt was made to achieve good recovery during the isolation of the radioactive steroids, and only the peak fractions were taken for further chromatography, because it was thought that radiochemical purity of the final material was the important consideration.

Stability of the [16-^{3}H]*progesterone*

Non-radioactive progesterone (Sigma) was purified by column chromatography, as described for the isolation [16-^{3}H]progesterone, and crystallized from the mobile phase. The dried crystals gave m.p. 121°.

[16-^{3}H]Progesterone, prepared and characterized as described above, except that the final specific activity was $3{\cdot}04\,\mu c/\mu g$., was dissolved in benzene (770 μg./ml., 2·35 mc/ml.) and kept at 0° for 3 months. From this solution 154 μg. was then added to 15·2 mg. of non-radioactive progesterone and the specific activity of the mixture determined by flow counter and u.v. absorption as $(3{\cdot}04\pm0{\cdot}08)\times10^{-2}\,\mu c/\mu g$. A portion of the mixture was now chromatographed on the same column system and the specific activity of the steroid estimated in the various fractions. The distribution of steroid and radioactivity was that of pure progesterone and the specific activity was equal in all fractions within the limits of experimental error. The mean specific activity of the two peak fractions was $(3{\cdot}01\pm0{\cdot}08)\times10^{-2}\,\mu c/\mu g$., indicating $(99\pm4)\%$ pure [16-^{3}H]progesterone after 3 months' storage. The peak fractions were pooled and the progesterone crystallized from the mobile phase. The crystals were washed with light petroleum (b.p. 40–60°) at room temperature and dried over silica gel *in vacuo* for 15 hr. at 60°. The crystals (4·91 mg.) had m.p. 120·5–121·5° and specific activity $(2{\cdot}9_3\pm0{\cdot}08)\times10^{-2}\,\mu c/\mu g$. This indicated that $(96{\cdot}5\pm4)\%$ pure [16-^{3}H]progesterone had been obtained after storage.

DISCUSSION

The yield of radioactivity as [16-^{3}H]progesterone from tritium gas was 3·6%. The reasons for this low yield have been discussed by Pearlman (1957*a*). The radioactive yield of [16-^{3}H]aldosterone from [16-^{3}H]progesterone was, from the results of the preliminary analysis, 2·9%. The overall yield

of radioactivity in the form of [16-^{3}H]aldosterone from tritium gas was 0·1%. The cheapness of tritium gas makes this low figure not a serious disadvantage. The preparation of [4-^{14}C]aldosterone in a similar manner, by incubating with a [4-^{14}C]intermediate such as [4-^{14}C]corticosterone, would be very expensive and would give maximum specific activities of about 0·03 μc/μg. As the secretion rate of this hormone by man is about 200 μg./day and the peripheral blood concentrations are about 0·03 μg./100 ml. of plasma (Ayres *et al.* 1957*a*) this material would be of too low a specific activity for most purposes. The overall yield of radioactivity from tritium to [16-^{3}H]-corticosterone is nearly 1%, which is satisfactory. The production of radioactive cortisol during the incubation is probably due to contamination with zona fasciculata tissue, which is unavoidable in the large-scale preparation of tissue. The specific activity of this compound is much lower than that of either corticosterone or aldosterone. This phenomenon of lower specific activity, which has been consistently observed when capsule strippings are incubated with either [16-^{3}H]- or [4-^{14}C]progesterone, and at different steroid-substrate concentrations, is as yet unexplained. It seems unlikely that it is due to high concentrations of cortisol or of precursors in the tissue before incubation. The use of [16-^{3}H]cortisol may have some advantages on the grounds of radiation safety in the study of the metabolism of this hormone in such conditions as human pregnancy. It could probably be made more efficiently by incubating [16-^{3}H]progesterone with whole adrenocortical tissue or by a microbiological process from the same radioactive steroid.

The synthetic preparation of [16-^{3}H]corticosterone and aldosterone from appropriate starting products might be achieved by routes similar to those employed in the preparation of [16-^{3}H]progesterone (Pearlman, 1957*a*). The synthesis of aldosterone (Schmidlin, Anner, Billeter & Wettstein, 1955) increases this possibility, especially as one of the steps involves hydrogenating a C-16-unsaturated compound. However, the steps are numerous from this point and the yield is low, especially of the naturally occurring (+)-aldosterone, which would have to be obtained for metabolic studies rather than the racemic mixture. In addition, the labile tritium at C-17 would have to be eliminated. Tritium-labelled adrenocortical hormones have been prepared by Fukushima & Gallagher (1952) by isotope-exchange procedures; the amounts of stably bound tritium incorporated were small, but still useful for metabolic experiments. Another route to the preparation of tritiated compounds may be found in the Wilzbach (1957) technique, which is very simple, requiring merely exposure of the compound to tritium gas. The tritiated product requires rigorous purification, as the formation of a variety of radioactive by-products may be expected. The isotopic labelling is of a random nature. The specific activity of the final product, although high, does not approach the order of radioactivity obtainable by synthetic methods.

The results reported here confirm the earlier findings that the zona glomerulosa produces a high ratio of aldosterone to cortisol compared with whole adrenal cortex (Ayres *et al.* 1956*a*). The isolation of crystalline radioactive aldosterone makes the identification of the preferentially produced compound more rigorous and confirms that the hormone obtained after incubation is being biosynthesized rather than merely released from the tissue into the surrounding fluid. They also confirm that progesterone can be an intermediate in the biosynthesis of aldosterone (Ayres *et al.* 1956*b*), which supports the conclusion of the one group (Rosenberg, Rosenfeld, Ungar & Dorfman, 1956) but not that of another (Wettstein, Kahnt & Neher, 1955).

The results of the experiment on the stability of [16-^{3}H]progesterone indicates that there is no significant radioactive disintegration when a solution of 2 mc/ml. in benzene is kept for 3 months at 0° in air, the [16-^{3}H]progesterone being then (99 ± 4) and $[96{\cdot}5 \pm 4)$% pure as determined by two methods. However, the slight indication of impurity from the latter result, although not quite significant, suggests that it may be better to store this steroid at a lower concentration. The stability of the radioactive adrenocortical hormones remains to be seen.

SUMMARY

1. [16-^{3}H]Progesterone (6·35 μc/μg.), prepared by the method of Pearlman (1957*a*), was incubated with capsule strippings of ox adrenal gland.

2. Crystalline [16-^{3}H]corticosterone, [16-^{3}H]-aldosterone and [16-^{3}H]cortisol were prepared from an extract of the incubation. The specific activities were $2{\cdot}6_2$, $1{\cdot}8_2$ and 0·76 μc/μg respectively.

3. The radiochemical purity of these steroids was established by various criteria.

4. Methods of assaying tritiated steroids by a flow counter and by means of *bremsstrahlung* emission are described.

5. The stability of the [16-^{3}H]progesterone has been examined and the purity found to be satisfactory after three months' storage.

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The Effects of the Hyaluronic Acid Complex on the Distribution of Ions

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Manery (1954) and Meyer & Rapport (1951) have suggested that hyaluronic acid, which is believed to be widely distributed in connective tissue, may have important effects on the distribution of ions within the body fluids. The present work was undertaken primarily to test this suggestion. Since there is evidence (Ogston & Stanier, 1950, 1952; Blumberg & Ogston, 1957*a*, *b*) that hyaluronic acid exists in synovial fluid and connective tissue as a complex with protein, it was decided to use this material, isolated from synovial fluid.

Although this preparation is chemically more complex than the polysaccharide isolated by Meyer & Palmer (1934) and others, the results obtained have also thrown light on its properties as a polyelectrolyte. Theoretical consideration of this aspect of the work leads to certain criticisms of the commonly used concept of 'ion-binding' (Klotz, 1953).

Ionic distributions have been commonly used for the estimation of extracellular volumes, and the general question of the steric exclusion of solutes from solutions of hyaluronic acid complex has been studied through the investigation of the distribution of the trisaccharide raffinose in solutions of the complex.

EXPERIMENTAL

Hyaluronic acid complex. This was prepared by the method of Ogston & Stanier (1950). The complex was washed on a sintered-glass ultrafilter of average pore diameter 1 μ by redispersion in wash fluid followed by refiltration. Six washes were carried out with 0·2N-NaCl solution, and six with water. Wash fluids were not buffered, so that ions extraneous to the experiments might not be introduced. Curtain (1955) showed that this variation in wash fluids was permissible.

Dialysis. Dialysis bags containing about 3 ml. of a solution of hyaluronic acid complex in water were dialysed at 4° in glass conical flasks against solutions containing the ions under study or raffinose. All solutions were saturated with chloroform as antiseptic. After 5–7 days the phases in

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equilibrium were sampled for analysis. Forty-two dialyses of the complex against solutions of NaCl were performed. The concentration of NaCl was varied between 18 and 145 mM. Comparative experiments with KCl, KSCN and $CaCl_2$ were also performed.

Variation of pH. The unbuffered NaCl solutions showed a range of pH 5·6–6·3. A higher range, pH 7·8–8·1, was obtained by the addition to the NaCl solutions of $NaHCO_3$ in such concentration that [HCO_3^- ion] = 0·32 [Cl^- ion], which follows approximately the relative concentration of the ions in serum (Gamble, 1954).

Determination of chloride. Chloride in the solutions was determined by potentiometric microtitration of weighed samples of solution containing about 300 μg. of chloride with standard $AgNO_3$ solution. The electrode system was Ag–unknown solution–0·1 N-NH_4NO_3 bridge–sat. KCl/AgCl–Ag. Mixing was given by the stationary electrodes while the vessel which contained the solution was rotated on a pneumatic turntable. Care was needed to ensure that annular concentration gradients were not set up in the viscous solution.

Solutions were made up to 1 ml. with water before titration, unless $NaHCO_3$ was present, when 0·1 N-HNO_3 was used to eliminate interference by HCO_3^- ions. Preliminary experiment showed that the hyaluronic acid complex did not interfere in the determination of chloride. The accuracy of this method was sufficiently high for the small differences in chloride concentration between the phases to be estimated with significance. The S.E.M. of four replicate determinations was about 0·1%.

Determination of thiocyanate. This ion was determined by the same method as that given above for chloride. The same order of accuracy was obtained.

Determination of bicarbonate. Bicarbonate was determined by volumetric measurement of CO_2 formed by acidification of weighed samples of solution with 0·1 N-HCl in a Warburg constant-volume-manometer apparatus. The apparatus was used in the standard manner, with special attention to attaining the highest possible accuracy with it. The samples yielded 100–150 μl. of CO_2; when it was necessary, the volume in the flasks was made up to approx. 3 ml. with CO_2-free water. The S.E.M. of duplicate determinations was about 0·3%, which is rather better than is usually obtained with the Warburg apparatus.

Determination of sodium. Sodium was determined flame photometrically with a Beckman model DU spectrophotometer with flame attachment as modified by Exley & Sproat (1958). Replicates of solution containing about 5 μg. of sodium were weighed into polythene weighing bottles, and diluted to 2 ml. with water which had been passed through a column of ion-exchange resin Amberlite IR-120-H so that its sodium concentration was less than 20 μg./l. Hyaluronidase (0·2 i.u.) was then added to each and the solutions were kept at 37° for 16 hr. in a water bath. The solutions were then diluted with the special 'low-sodium' water to about 5 g. by wt. Hyaluronic acid complex present was then so degraded and diluted that the viscosities of the solutions were not sensibly different from that of water, and sodium could be determined by direct comparison with standards in the flame photometer. The photoelectric signal of the instrument was directly amplified and measured with a pen recorder. With experience, only four replicates were necessary to obtain an S.E.M. of 0·1–0·2%.

Determination of calcium. Calcium was determined flame photometrically in preparations of hyaluronic acid complex with the same method of degradation and dilution of the sample as was used for sodium. The final concentration of calcium in the solutions analysed was about 8 μg./g. The accuracy was about 2%.

Determination of hyaluronic acid complex. The concentration of the complex was determined from the difference between the dry weight of a weighed sample of solution in water (after heating at 102–104° for 2 hr.) and the weight of the same sample after ignition.

Determination of raffinose. The sugar was determined colorimetrically by the method of Kulka (1956), after deproteinization of solutions with HPO_3. A small correction was necessary for the colour developed from the complex.

Direct determination of the equivalence of hyaluronic acid complex. The equivalence of the complex was directly determined by titration with cetylpyridinium chloride solution (Scott, 1956) of buffered solutions of the complex. Two buffers were used. The first, of pH 7·8–7·9, contained 0·1 N-$NaHCO_3$ and 0·01 N-Na_2SO_4. The second contained Na_2HPO_4 and NaH_2PO_4 at pH 7·0 and had ionic strength 0·175, and Na_2SO_4 was dissolved in it to 0·01 N.

Hyaluronic acid complex solution (0·5 g.) was weighed out, and about 2 ml. of buffer solution added. An excess of cetylpyridinium chloride solution of known concentration was added slowly with mixing and the solution allowed to stand for 15 min. The SO_4^{2-} ion present assisted the formation of a compact precipitate, which was removed by centrifuging. The excess of reagent was then estimated spectrophotometrically at 260 mμ.

The cetylpyridinium chloride was twice recrystallized from water; the equivalence of its solution (3·85 m-equiv./l.) was determined by titrating its chloride content by the method described.

THEORETICAL

The interaction of ions with a polyelectrolyte may be treated in two ways: (i) by the description of the system as an equilibrium between the polyelectrolyte, the interacting ion and a polyelectrolyte–ion complex; or (ii) by the statement of the parameters of a Donnan equilibrium. The first method is potentially more powerful, in that it can describe other forms of interaction supplementing electrostatic interaction, but it suffers from the disadvantage that it implies that the interaction can be forced to completion by increasing the ionic concentration. This is not necessarily so, as the interacting ion can only be introduced into solution together with ions of opposite sign, which also enter the interacting system. The second method does not take into account forces of interaction other than electrostatic forces, but it is mathematically simpler, and has the advantage of describing the distributions of ions of both signs. Adamson (1952) has pointed out that the two treatments are thermodynamically consistent, differing only in their formulation of the standard states.

It is now recognized (Overbeek, 1956) that, for the univalent ions of the salt MA, the simple statement of the Donnan equilibrium

$$[M^+][A^-] = [M^+]'[A^-]' \qquad (1)$$

is not valid in dilute solutions of a polyelectrolyte with a high surface potential, and the true equilibria have been

described (i) by the introduction of empirical activity coefficients, (ii) by the description of deviations as 'ion-binding', (iii) by the recalculation of the Donnan equation without the use of the mathematical approximations of Debye and Hückel. Klaarenbeek (1946) has calculated and experimentally verified that the Donnan equilibrium in a solution of a colloid that bears charges only of negative sign can be described in terms of two parameters, which we may call E, the equivalence of the colloid in m-equiv./g., and α_-, the fraction of the colloidal equivalence which is neutralized by a deficiency in the solution of ions of the same sign as the colloid. α_- should be independent of ionic strength in a given system, and lies between 0 and 0·5. It will be apparent that these parameters can also be used to describe systems where other types of interactions occur. Then for a negative colloid

$$\left.\begin{aligned}\Delta[A^-] &= -\alpha_- E\\ \Delta[M^+] &= (1-\alpha_-)\,E\end{aligned}\right\} \qquad (2)$$

where $\Delta[A^-]$ and $\Delta[M^+]$ are the changes, in m-equiv./g. of colloid, in the concentrations of the anion and cation respectively in the colloid phase following from interactions of the ions with the colloid.

This treatment can be extended to solutions of colloids bearing charges of both signs. The proteins are an example of this type of colloid. The charge on an amphoteric colloid can be considered as occurring on a large number of equally charged zones of both signs. These charges either may interact with adjacent charges of opposite sign, so that the charge is in some part internally neutralized, or may induce local Donnan equilibria. Then a solution of 1 g. of a colloid can be considered to contain n_1 zones of unit negative charge and n_2 zones of unit positive charge, of which n_3 zones of each charge interact totally with each other, so that (n_1-n_3) negative zones and (n_2-n_3) positive zones induce local Donnan equilibria.

At any one of the negative areas, j, which induces a Donnan equilibrium,

$$\left.\begin{aligned}{}_j\Delta[A^-] &= -{}_1\alpha_-\,{}_je_-\\ {}_j\Delta[M^+] &= (1-{}_j\alpha_-)\,{}_je_-\end{aligned}\right\} \qquad (3)$$

where ${}_je_-$ is the equivalence of area j, which is equal to the equivalence of each of the other negative areas. The gross negative equivalence $E_- = \Sigma_j e_- = n_1 e_-$. Of this gross negative equivalence, the quantity $E_i = n_3 e_-$ will interact internally with positive zones. Then the negatively charged areas will induce as a sum shifts in the concentrations of M^+ and A^-, as given by

$$\Delta[A^-]_1 = \Sigma_n^n(-{}_j\alpha_-e_-) = -\alpha_-(E_- - E_i), \qquad (4)$$

and

$$\Delta[M^+]_1 = (1-\alpha_-)\,(E_- - E_i). \qquad (5)$$

where α_- is defined by equation (4) and will lie between 0 and 0·5.

We may similarly write for the Donnan shifts due to the positive charges, of total equivalence $E_+ = n_2 e_+$,

$$\Delta[A^-]_2 = (1-\alpha_+)\,(E_+ - E_i), \qquad (6)$$

$$\Delta[M^+]_2 = -\alpha_+(E_+ - E_i). \qquad (7)$$

Equations (4) and (6) and equations (5) and (7) may be summed to give the following identities, which describe the observed total shifts in ionic concentrations:

$$\Delta[A^-] = E_+(1-\alpha_+) - E_-\alpha_- - E_i(1-\alpha_+-\alpha_-), \qquad (8)$$

and

$$\Delta[M^+] = E_-(1-\alpha_-) - E_+\alpha_+ - E_i(1-\alpha_+-\alpha_-). \qquad (9)$$

Several remarks can be made on these equations: (i) that, when $\alpha_- = \alpha_+ = 0{\cdot}5$, $-\Delta[A^-] = \Delta[M^+] = 0{\cdot}5(E_- - E_+)$, which is the classical statement of the Donnan equilibrium for negative colloid ($E_- > E_+$) due to its net charge ($E_- - E_+$). This would be expected to hold when the ionic strength is very low so that electrostatic shielding between charged areas on the colloid is negligible; (ii) that when the ionic strength is high, so that charged areas, shielded from each other, tend to induce Donnan equilibria independently of each other, then E_i becomes disappearingly small and $\Delta[A^-]$ will increase and may reach positive values.

This phenomenon, for example, of anion concentrations in solutions of proteins, showing progressively increasing deviations from the predictions of a Donnan function with increasing ionic strength, is well known and is termed 'ion-binding'. The deviations are commonly used as a measure of the number of positive 'sites' to which the anion is 'bound', i.e. as a method of investigation of the fine structure of colloids. It will be apparent from equations (8) and (9) that a knowledge of $\Delta[A^-]$ and $\Delta[M^+]$ from ionic-distribution experiments is not sufficient to permit the calculation of E_- and E_+.

In the experiments described, apparent values of α_- have been computed from equation (2), and interpretation of the variation of α_- has been restricted to qualitative statements of the zwitterion character of the hyaluronic acid complex.

RESULTS

Distribution of sodium salts in solutions of hyaluronic acid complex

Competition between chloride and bicarbonate ions. The statement of the parameters of a Donnan distribution will describe the changes in anion and cation concentrations in the colloidal phase, but will not distinguish between ions of the same sign and charge. An ion may preferentially displace another ion which is electrically identical from an ionic double layer because of differences other than charge. Two ions are said to behave ideally if the changes in their concentrations in the colloid phase are proportional to their concentrations in the diffusate. Table 1 shows the determined deficiencies in Cl^- and HCO_3^- ions in the colloid phase in two dialyses of hyaluronic acid complex solutions against solutions containing NaCl and $NaHCO_3$. The concentration deficiencies of each ion are expressed as a percentage of their concentrations in the dialysing solution. In each dialysis, the percentage deficiency of HCO_3^- ion was the same, within the limits of experimental error, as that of Cl^- ion. It was therefore proper, knowing the ratio between the concentrations of the two ions in the dialysing solutions as prepared, to calculate the total deficiency of anion, $\Delta[A^-]$, for all dialyses where both Cl^- and HCO_3^- ions were present, as

$$\Delta[A^-] = \frac{[A^-]}{[Cl^-]}\,\Delta[Cl^-]. \qquad (10)$$

Table 1. *Donnan expulsion of chloride and bicarbonate ions from hyaluronic acid complex solutions*

C is the concentration of an ion in the dialysing solution. Percentage deficiency is the concentration deficiency of an ion in the colloid phase, expressed as a percentage of its deficiency in the dialysing solution. S.E. is the standard error of the percentage deficiency.

Dialysis no.	Ion	*C* (m-equiv./kg.)	Percentage deficiency	S.E.
43	Cl^-	10·55	2·2	0·4
	HCO_3^-	3·62	1·9	0·4
44	Cl^-	10·56	3·2	0·1
	HCO_3^-	10·65	2·9	0·4

Table 2. *Changes in ionic concentrations in solutions of hyaluronic acid complex and sodium salts, and calculation of coefficient* α_-

[NaA] is the concentration of sodium salts. Experimental values of the changes in concentration of Cl^- and Na^+ ions, $\Delta[Cl^-]$ and $\Delta[Na^+]$, are in units of m-equiv./kg. of solution, and have a negative sign when the ion is in a deficiency in the colloid phase. The standard errors of $\Delta[Cl^-]$ and $\Delta[Na^+]$ are shown in parentheses. In the last 16 rows of results, HCO_3^- ion was present, and $\Delta[A^-] = 1{\cdot}32\ \Delta[Cl^-]$. α_- is calculated from equation (2), $\Delta[A^-] = -\alpha_- E$, where E is estimated from equation (11), and $\Delta[A^-]$ is in units of m-equiv./g. of hyaluronic acid complex (HAC).

pH	Concn. of HAC (%, w/w)	[NaA] (m-moles/kg.)	$\Delta[Cl^-]$	$\Delta[Na^+]$	α_-
5·6	0·34	144	2·4 (0·55)	4·3 (0·59)	−1·42
5·8	0·31	143	2·2 (0·22)	3·9 (0·34)	−1·22
5·7	0·22	143	0·7 (0·24)	1·4 (0·40)	−0·59
5·6	0·14	144	0·7 (0·23)	1·4 (0·42)	−1·00
5·7	0·16	141	0·6 (0·15)	1·6 (0·48)	−0·70
5·7	0·12	142	0·7 (0·12)	1·1 (0·45)	−1·07
5·8	0·20	96	0·3 (0·14)	1·1 (0·36)	−0·26
5·6	0·22	96	0·7 (0·21)	1·7 (0·40)	−0·64
5·6	0·19	48	−0·3 (0·18)	1·0 (0·18)	0·32
5·8	0·21	49	−0·2 (0·08)	1·0 (0·21)	0·17
5·9	0·39	139	1·9 (0·15)	3·6 (0·48)	−1·02
5·7	0·17	138	0·7 (0·31)	—	−0·76
5·8	0·40	47	−0·67 (0·07)	1·9 (0·26)	0·29
5·8	0·14	46	−0·15 (0·04)	—	0·19
6·1	0·31	136	0·3 (0·15)	2·6 (0·24)	−0·14
6·0	0·32	136	−0·1 (0·14)	—	0·05
5·9	0·27	46	−0·69 (0·15)	—	0·43
6·1	0·28	46	−0·61 (0·06)	—	0·32
5·8	0·29	19	−0·88 (0·08)	—	0·52
6·3	0·28	20	−1·10 (0·10)	1·53 (0·13)	0·51
6·2	0·16	136	−0·2 (0·10)	—	0·18
6·1	0·24	136	−0·1 (0·07)	—	0·06
5·9	0·22	46	−0·30 (0·06)	—	0·23
6·1	0·17	46	−0·20 (0·03)	—	0·17
5·9	0·16	19	−0·33 (0·05)	—	0·34
5·9	0·16	19	−0·38 (0·06)	—	0·32
7·8	0·40	142	−1·6 (0·14)	—	0·40
7·8	0·40	140	−1·4 (0·13)	—	0·34
8·0	0·14	140	−0·2 (0·09)	—	0·15
8·0	0·13	139	−0·3 (0·10)	—	0·22
7·8	0·32	138	−1·9 (0·13)	2·0 (0·11)	0·59
8·0	0·30	136	−1·3 (0·14)	—	0·41
8·1	0·28	45	−0·51 (0·03)	—	0·17
8·0	0·29	46	−0·93 (0·07)	—	0·30
7·8	0·30	19	−0·87 (0·03)	—	0·29
7·8	0·30	18	−0·79 (0·04)	2·73 (0·07)	0·27
7·9	0·16	135	−0·4 (0·14)	—	0·23
7·9	0·15	135	−0·4 (0·17)	—	0·24
7·9	0·17	45	−0·45 (0·05)	—	0·26
8·0	0·15	45	−0·35 (0·04)	—	0·22
7·9	0·16	19	−0·36 (0·03)	—	0·22
7·8	0·16	18	−0·38 (0·06)	—	0·23

Equivalence of the hyaluronic acid complex. The analytical data describing the dialyses performed are given in columns 1–5 in Table 2. After transforming $\Delta[Cl^-]$ and $\Delta[Na^+]$ to units of m-equiv./g. of complex, and calculating $\Delta[A^-] = 1{\cdot}32\ \Delta[Cl^-]$ for the dialyses where HCO_3^- ion was present (pH 7·8–8·1), values were calculated of the equivalence of the complex/g., $E = \Delta[Na^+] - \Delta[A^-]$, for the 16 dialyses where both $\Delta[Na^+]$ and $\Delta[A^-]$ were determined. These values of E were linearly correlated with pH and [NaA], the millimolal concentration of sodium salts. There was found to be no significant correlation between E and [NaA], but the correlation between E and pH was highly significant, the relationship being

$$E = 0{\cdot}37\ \text{pH} - 1{\cdot}57, \qquad (11)$$

where E is in units of m-equiv./g. of complex. It is realized that the true form of this relationship is likely to be that of a titration curve, and not linear. However, the results were not sufficiently accurate to permit the fitting of any particular titration curve, and the linear form was sufficient to demonstrate a correlation between E and pH and to allow estimates of E to be made for the calculation of α_-. The approximation is good enough to demonstrate the polyelectrolyte character of the complex, and the fitting of a more plausible form of function would not affect the conclusions drawn.

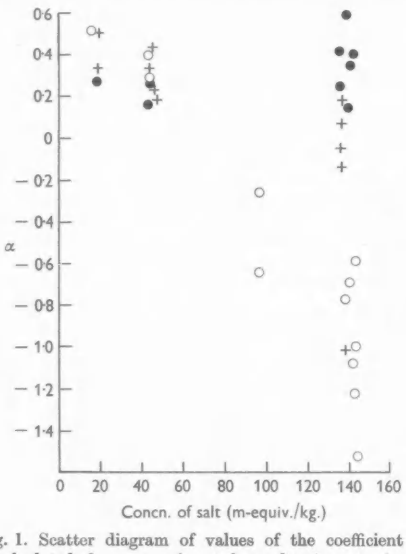

Fig. 1. Scatter diagram of values of the coefficient α_- calculated from experimental results (see text) and plotted against concentration of salt. Some experimental values have been omitted to avoid crowding of the diagram. ○, pH 5·5–5·8; +, pH 5·9–6·3; ●, pH 7·8–8·1.

Experimental values of α_-, and its correlation with pH *and salt concentration.* From values of $\Delta[A^-]$ in the consistent units of m-equiv./g. of complex, and estimates of E from equation (11), values of α_- could be calculated with equation (2). These values are shown in the last column of Table 2, and are plotted against [NaA] in Fig. 1. In this figure different symbols are used to indicate pH ranges. At low pH values, and higher values of [NaA], negative values of α_- occur. Statistical correlation of these values showed that, in the range pH 7·8–8·1, α_- was independent of pH and ionic strength, being constant at 0·3. In the range pH 5·5–6·3, the data can be fitted to the regression equation

$$\alpha_- = -1{\cdot}39 \times 10^{-1}\,[\text{NaA}] - 9{\cdot}46 \times 10^{-1}\,(\text{pH}) + 2{\cdot}22 \times 10^{-2}\,(\text{pH})\,[\text{NaA}] + 6{\cdot}19. \qquad (12)$$

The coefficients of equation (12) are significant at the 0·1% level. The partial correlations are probably not linear, as this regression equation implies, but the experimental scatter does not permit more accurate curve fitting. The high significance of the coefficients in equation (12) indicates, however, that it can be stated qualitatively that, in this pH range, α_- does not conform to the Klaarenbeek prediction that it will lie between 0 and 0·5, and will be independent of ionic strength. Rather, in this range, pH 5·5–6·3, it decreases and reaches negative values with increasing ionic strength, the effect being more pronounced with decreasing pH. In the range pH 7·8–8·1, $\alpha_- = 0{\cdot}3$, and is independent of ionic strength, and therefore conforms with the predictions of Klaarenbeek.

Distribution of other electrolytes. The results of comparative experiments which examined the independent distributions in solutions of hyaluronic acid complex of the ions of NaCl, KCl, KSCN and $CaCl_2$ are shown in Table 3. Comparison of the results for NaCl, KCl and KSCN shows that these salts are all distributed with a value of α_- of 0·3. The value of α_- in $CaCl_2$ solutions, at a mean of 0·06, is, as would be expected for a salt of Cl^- ion with a bivalent ion, lower than 0·3.

Equivalence of hyaluronic acid complex; titration with cetylpyridinium chloride. The titration of samples of the complex with a standard solution of cetylpyridinium chloride provided an independent estimate of the equivalence of the complex. The results are shown in Table 4. At both pH 7·0 and 7·8, four samples of the complex were found to have equivalence values which were constant at 3·2 m-equiv./g. One of the samples was prepared by Mr T. F. Sherman by the electrodialysis of synovial fluid (Ogston & Sherman, 1958). Another had been extracted with the chelating agent ethylenediaminetetra-acetic acid (EDTA) to remove metals which might be masking acidic groups. This

Table 3. *Changes in ionic concentrations in solutions of hyaluronic acid complex and various electrolytes, and calculation of* α_-

For explanation see Table 2. S.E. of $\Delta[A^-] = 0{\cdot}03$–$0{\cdot}06$.

Salt	pH	Concn. of HAC (%, w/w)	Concn. of salt (m-moles/kg.)	$[A^-]$ (m-equiv./kg. of soln.)	α_-
NaCl	5·6	0·20	21	0·36	0·36
	5·7	0·18	21	0·25	0·26
KCl	5·7	0·19	22	0·29	0·28
	5·7	0·19	22	0·32	0·31
KSCN	5·6	0·19	20	0·35	0·36
	5·8	0·19	20	0·32	0·29
$CaCl_2$	5·7	0·18	17	0·04	0·04
	5·6	0·21	17	0·08	0·08

Table 4. *Equivalence of samples of complex by cetylpyridinium chloride titration*

Duplicate results are shown for each sample. Samples 7 and 8 were prepared by ultrafiltration of synovial fluid. Sample ED was prepared by electrodialysis of synovial fluid. Sample 7V is sample 7, extracted with ethylenediaminetetra-acetic acid by dialysis and then dialysed against distilled water.

Sample	pH	Equivalence (m-equiv./g. of complex)
7	7·0	3·11
		3·23
8	7·8	3·23
		3·26
ED	7·8	3·29
		3·16
7V	7·0	3·19
		3·29

Table 5. *Distribution data for raffinose in solutions of hyaluronic acid complex*

'Outside' and 'inside' refer to the diffusate outside the dialysis bag and the colloid phase inside the bag.

Concn. of raffinose: Outside (g./l.)	Inside (% of outside concn.)	Concn. of complex (g./kg.)	Excluded vol. (ml./g. of complex)	r_E (Å)
5·0	98·2	2·8	6·6	13·2
5·0	98·4	2·8	5·7	12·3
5·0	98·4	2·7	5·9	12·5
5·0	98·3	2·7	6·3	12·9
2·4	98·7	2·8	4·6	11·1
2·4	97·9	2·8	7·5	14·1
2·4	98·5	2·7	5·6	12·1
2·4	98·1	2·7	7·0	13·7
		Mean	6·2	12·7

value of 3·2 m-equiv./g. does not agree with the value of 1·3 m-equiv./g. at pH 7·8, which is given by the equivalence of sodium salts interacting with the complex (equation 11). This suggests that some other cation is present in the ultrafiltered preparations of the complex which can be displaced by the cetylpyridinium ion, but masks acidic radicals so that they do not interact with univalent ions.

Determination of calcium in samples of complex prepared by ultrafiltration. Calcium has been reported in samples of hyaluronic acid complex prepared by ultrafiltration (Ogston & Stanier, 1952). It therefore appeared possible that its presence might account for the disparity found between estimates of the equivalence by the two independent methods. Analysis of two preparations of the complex gave values of 1·86 and 1·78 m-equiv. of calcium/g. of complex. These figures lead to a total equivalence of 3·1 m-equiv./g. estimated to be interacting with ions, which shows good agreement with the equivalence determined directly to be 3·2 m-equiv./g.

Distribution of raffinose in solutions of hyaluronic acid complex. Blumberg & Ogston (1956) showed, using sedimentation techniques, that the domain of the hyaluronic acid complex random coil was permeable to solutes. In these experiments the observed distributions of raffinose were checked against the expected steric exclusion of solutes due to the threads of the random coil of the complex. The length of the disaccharide repeating unit of the complex (Meyer, 1953) may be taken to be about the same as that of cellulose, 10·25 Å (Sponsler & Dore, 1926). The length of polysaccharide chain in 1 g. of complex may then be calculated to be $1{\cdot}23 \times 10^{14}$ cm., and taking a cylindrical thread model for the complex, the specific volume of the complex at 0·69 (Ogston & Stanier, 1950) can be used to derive a mean radius of the complex thread of 4·2 Å. A solute of molecular radius r Å will therefore be sterically excluded by 1 g. of complex from a volume of

$$(r+4{\cdot}2)^2 \times 10^{-16} \times 1{\cdot}23 \times 10^{14} \text{ cm.}^3,$$

$(r+4{\cdot}2)$, which we may call r_E, the radius of exclusion, can therefore be estimated from distribution data. The experimental results are shown in Table 5. The mean excluded volume to raffinose

was 6·2 ml./g. of complex, and $r_g = 12{\cdot}7$ Å. This gives 8·5 Å as an estimate of the effective radius of raffinose; its molecular dimensions would be expected to be about 15 Å × 5 Å.

DISCUSSION

Equivalence of the hyaluronic acid complex. The equivalence of the complex has been determined by direct titration to be 3·2 m-equiv./g., and a balance has been struck with cations and anions in solution which supports this figure. It is, however, surprisingly high. The equivalence of the polysaccharide carboxyls on the complex, assuming one glucuronic acid residue to every *N*-acetylglucosamine residue, and 25 % of protein in the complex, is 2·0 m-equiv./g., and if the unconfirmed analysis of Ogston & Stanier (1952) is taken, it may be as high as 2·2 m-equiv./g. This determination therefore demands that the equivalence of the protein in the complex is about 4·0 m-equiv./g., which seems too high. It seems that the chemical constitution of the complex requires further examination before light can be shed on this question.

Polyelectrolyte character of hyaluronic acid complex. The theoretical treatment which has been presented above of the Donnan distributions around amphoteric polyelectrolytes can be used to derive qualitative statements of the polyelectrolyte character of the complex. The experimental knowledge of the variables

$$\Delta[Na^+] - \Delta[A^-] = E_- - E_+$$

is not sufficient to solve equation (8) or (9) for any parameters of the molecule without unwarranted assumptions. Qualitatively it can be stated that in the range pH 7·8–8·1 the independence of α_- from ionic strength means that, in this range of pH, the complex acts as a colloidal particle bearing charges of only one sign. With decreasing pH the charge on the complex interacting with the ions of sodium salts decreases as might be expected for the complex of a polyacid and a protein above its isoelectric point. In the range pH 5·5–6·3 the anion is not always in a deficiency in the colloidal phase, and apparent 'ion-binding' has been observed, more particularly at low pH and high ionic strength. This effect is reflected in negative values of α_- under these circumstances. The comparison of the results for sodium chloride with those for other univalent ions provides no evidence of specific interactions. The variations in α_- for sodium chloride are explained under the theory given as indicating zwitterion character of the complex at low pH values. At low ionic strengths the effect is obscured through the interaction of the variously charged areas on the complex which results from incomplete electrostatic screening. The partial equivalences of the zwitterion under any one set of conditions necessarily cannot be calculated from the data. The zwitterion character of the complex at low pH values is to be expected from its constitution. The statistical correlation of α_- against pH and ionic strength (equation 12) suggests that the amphoteric nature of the complex will not be important at physiological pH values. This is supported by the observation that the total equivalence as determined by direct titration is the same at pH 7·0 as at pH 7·8.

Use and abuse of the concept of 'ion-binding'. It has been common in describing the interactions of ions with polyelectrolytes for deviations from the predictions of the simple Donnan function (equation 1) to be denoted as being a result of the 'binding' of ions to the polyelectrolyte, and distributions are described in terms of a sum of the 'Donnan equilibrium' and the quantity of one of the ions which is 'bound' to the polyelectrolyte. The distinction is drawn between 'specific' and 'non-specific' ion-binding, where the first term is applied to cases where one type of ion shows highly individual effects. In these cases, where precipitation or stoicheiometric interaction is observed, the chemical reaction which the term 'binding' connotes is obviously occurring, and these cases will not be discussed further.

However, in cases of non-specific ion-binding, where it is recognized (Klotz, 1953) that the effects are due to electrostatic 'binding' of ions to charged 'sites' on the polyelectrolyte, the use of the terminology of ion-binding in the description of ionic distributions is more open to criticism. In some cases its use can be criticized as being no more than clumsy, as in such cases where 'binding' is small, and constant with ionic strength (e.g. Farber & Schubert, 1957), where the statement of its occurrence is a result of the inadequacy of equation (1) as a Donnan function. Similarly, in describing the interactions of ions with amphoteric polyelectrolytes, such as the proteins, the statement of a degree of 'ion-binding' is defensible, if clumsy, but only if the term 'ion-binding' is recognized as having been defined to mean the empirically determined deviations from the predictions of an arbitrary law. However, if a pictorial meaning is given to the measured amounts of 'binding', and the chemical connotation of the word is transferred to it, as often has been done, fallacious interpretations of the results easily follow.

Equations (8) and (9) show that in the general case fine parameters of a colloid cannot be deduced from distribution experiments, and it will be apparent that the deduction of the 'number of binding sites' on a colloid must imply two assumptions: (i) that $E = E_- - E_+$, and (ii) that $\alpha_- = 0{\cdot}5$ and $\alpha_+ = 0{\cdot}0$. Nor must it be thought that alter-

native methods of measuring 'binding', such as the electrometric method of Scatchard, Sheinberg & Armstrong (1950) can circumvent this difficulty. These methods implicitly measure the deviations from the predictions of equation (1) in that they employ the same assumption on which equation (1) is based, that the ionic activity coefficients are the same in the two equilibrium phases, and define 'free' ion by this criterion. It is suggested that, in recognition of the general identity of Donnan equilibria and 'non-specific ion-binding' effects, the latter term might be conveniently discarded as introducing connotations which are improper and have proved dangerous, that 'non-specific ion-binding' is a chemical rather than an electrostatic phenomenon.

Interactions of calcium. The occurrence of calcium in preparations of hyaluronic acid complex which have been washed 12 times with sodium chloride solutions and with water shows that calcium is tightly associated with the complex, and cannot be easily displaced from that association by high concentrations of salts of univalent ions. This tight association is confirmed by the value $\alpha_- = 0{\cdot}06$, measured for the distribution of calcium chloride in solutions of the complex. (There is no evidence on whether this association is strengthened by forces of interaction other than the electrostatic; it can be qualitatively attributed to the higher place in the Hofmeister series which is normally held by bivalent ions.) It would therefore be expected that hyaluronic acid in physiological systems would be largely associated with Ca^{2+} ions, and that little residual charge would remain to interact with other ions, and to influence their distributions. The presence of hyaluronic acid in tissues would not therefore be expected to introduce any sensible error into the measurement of extracellular space from the distributions of such ions as Na^+ and Cl^-. This conclusion is supported by the work of Ropes, Bennett & Bauer (1939), on synovial fluid, who showed that the distribution only of Ca^{2+} ion was influenced by the presence of 'mucin'. The balance obtained between the equivalence of the complex as determined by titration with cetylpyridinium chloride and that accounted for interactions of ions, including Ca^{2+}, suggests that the calcium found in the preparations of the complex is not chemically integral with it, in that the calcium is displaceable by the cetylpyridinium ion.

Distribution of raffinose. The approximate concordance between the distributions of raffinose observed and the requirements of steric exclusion by a geometrical model of the complex as a cylindrical thread supports the conclusion of Blumberg & Ogston (1956) that the domain of the random coil of the complex is permeable to solutes. Further support is forthcoming from the ionic-distribution experiments, where there was no evidence that the steric exclusion was higher than that due to the specific volume of the complex, which was negligible in these experiments. The error in determinations of extracellular space which use raffinose distributions should be no greater than 1–2 %.

SUMMARY

1. The distribution of sodium chloride in solutions of the hyaluronic acid complex have been examined. At low pH values and at ionic strengths approaching the physiological, anomalous distributions have been observed which could be characterized as following 'binding' of Cl^- ion.

2. The theory of the distributions of univalent ions around an amphoteric polyelectrolyte has been briefly treated, and it has been shown that distribution experiments cannot provide sufficient information to permit the derivation of parameters of the polyelectrolyte other than its net equivalence. On the basis of this theory, the use of the concept of 'ion-binding' is discussed and criticized.

3. Sodium chloride-distribution experiments have been qualitatively interpreted as indicating that hyaluronic acid complex exhibits zwitterion characteristics at values below about pH 6·1.

4. Brief studies of the distributions of potassium chloride and potassium thiocyanate in solutions of the complex did not show any differences from the distributions of sodium chloride.

5. The distribution of calcium chloride in solutions of the complex was briefly examined. With the observation of the occurrence of calcium in washed preparations of the complex, this showed that Ca^{2+} ion was tightly associated with it, and that it can be expected that hyaluronic acid would be expected to be physiologically associated with Ca^{2+} ion. The complex should not introduce any error into determinations of 'sodium space' and 'chloride space'.

6. The direct titration of the complex with solutions of cetylpyridinium chloride consistently gave estimates of the equivalence of the complex at 3·2 m-equiv./g., which is surprisingly high.

7. The distribution of raffinose in solutions of the complex was approximately consistent with the steric requirements of a thread model for the complex. The domain of the random coil of the acid is thus confirmed as being permeable to solutes. The error in determinations of 'raffinose space' due to this effect should not exceed 2 %.

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The Isolation of 16β-Hydroxyoestrone and 16-Oxo-oestradiol-17β from the Urine of Pregnant Women

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While attempting to follow up the suggestion of Marrian & Bauld (1955) that 16-oxo-oestradiol-17β (3:17β-dihydroxyoestra-1:3:5-trien-16-one) might be the common metabolic precursor of oestriol (oestra-1:3:5-triene-3:16α:17β-triol) and 16-*epi*oestriol (oestra-1:3:5-triene-3:16β:17β-triol) Watson & Marrian (1955) reported the detection of a ketonic Kober chromogen in extracts of the urine of pregnant women which they provisionally identified as the sought-for 16-oxo-oestradiol-17β solely on partition-chromatographic and countercurrent-distribution evidence. Shortly afterwards Levitz, Spitzer & Twombly (1956) reported the detection by reverse isotope dilution of radioactive 16-oxo-oestradiol-17β in the urine of subjects after the administration of [16-^{14}C]oestradiol-17β ([16-^{14}C]-oestra-1:3:5-triene-3:17β-diol).

The following year the ketonic Kober chromogen detected in pregnancy urine by Watson & Marrian (1955) was isolated from the same source as a crystalline solid by Marrian, Watson & Panattoni (1957), and evidence was presented that the isolated product probably consisted of 16α-hydroxyoestrone (3:16α-dihydroxyoestra-1:3:5-triene-17-one) and a small proportion of 16-oxo-oestradiol-17β. It was furthermore suggested that the latter might have been an artifact produced from the former by rearrangement during the extraction of the phenolic fraction of the urinary extract by aqueous alkali from ether. The major component of the isolated product was subsequently identified with certainty as 16α-hydroxyoestrone by Marrian, Loke, Watson & Panattoni (1957), who also obtained evidence which apparently confirmed the earlier suggestion that the minor component in the product had been artifactually formed by rearrangement of 16α-hydroxyoestrone in alkaline solution. This evidence led the authors to conclude that pregnancy-urine extracts contain no 16-oxo-oestradiol-17β other than that which is formed artifactually in this way, and, because of this conclusion and of the difficulty in separating mixtures of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β by crystallization they queried the validity of the claim of Levitz *et al.* (1956) to have detected 16-oxo-oestradiol-17β in urine extracts, although the method of extraction used by the latter authors seemed to exclude the possibility of artifactual formation of 16-oxo-oestradiol-17β.

Marrian, Watson & Panattoni (1957) and Marrian, Loke, Watson & Panattoni (1957) suggested that 16α-hydroxyoestrone formed from

oestrone (3-hydroxyoestra-1:3:5-trien-17-one) by 16α-hydroxylation might be the metabolic precursor of oestriol, and that similarly 16β-hydroxyoestrone (3:16β-dihydroxyoestra-1:3:5-trien-17-one) might be the precursor of 16-*epi*oestriol. At that time these suggestions were entirely speculative, but they appeared to be sufficiently plausible to justify an intensive search in pregnancy urine for 16β-hydroxyoestrone.

This search has now been successfully concluded, and it has also resulted in the isolation from pregnancy urine of substantial amounts of 16-oxo-oestradiol-17β by methods which as far as can be determined exclude the possibility that the latter was formed artifactually from either 16α-hydroxyoestrone or 16β-hydroxyoestrone. Accordingly, the earlier criticisms of the work of Levitz *et al.* (1956) are now unreservedly withdrawn.

RESULTS

Detection of 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β in pregnancy-urine extracts

Early attempts to separate 16β-hydroxyoestrone [prepared synthetically by the method of Biggerstaff & Gallagher (1957)], 16α-hydroxyoestrone and 16-oxo-oestradiol-17β by paper chromatography with systems of the Bush (1952) type by using aqueous methanol as stationary phase were entirely unsuccessful. However, acting on a suggestion made to us by Dr T. F. Gallagher of the Sloan-Kettering Institute, New York, we tried the system formamide/chloroform and obtained excellent separation of the three α-ketols from one another.

When ketonic–phenolic fractions, prepared from enzymically-hydrolysed pregnancy urine by the method of Marrian, Watson & Panattoni (1957), were chromatographed on paper in the formamide/chloroform system spots corresponding in mobilities to 16α-hydroxyoestrone and 16-oxo-oestradiol-17β were found, but nothing corresponding to 16β-hydroxyoestrone was detected. The possibility was therefore considered that any 16β-hydroxyoestrone which might originally have been present in the urine had undergone rearrangement to 16-oxo-oestradiol-17β during the working-up process. In particular the possibility was considered that rearrangement might have occurred during the 48 hr. incubation at pH 4·8 and 37° involved in the enzymic hydrolysis of the urine, during the Girard separation of the ketonic fraction or during the 10 min. exposure to alkali involved in the separation of the phenolic fractions. In experiments with synthetic 16β-hydroxyoestrone no rearrangement to 16-oxo-oestradiol-17β, as judged by paper chromatography, was observed after incubation in aqueous solution at pH 4·8 and 37° for 48 hr. or after standing overnight at room temperature in ethanolic acetic acid. However, complete rearrangement to 16-oxo-oestradiol-17β was found to occur after the 16β-hydroxyoestrone had stood for 10 min. at room temperature in N-sodium hydroxide solution.

In view of these findings a ketonic neutral-phenolic fraction of urine was prepared without the use of alkali and chromatographed on a Celite column. A portion of the fraction from the column expected to contain the α-ketolic oestrogen derivatives was chromatographed on paper and spots corresponding in their mobilities to 16α-hydroxyoestrone, 16β-hydroxyoestrone and, unexpectedly, 16-oxo-oestradiol-17β were detected. Areas of paper corresponding to these spots were cut out from a second chromatogram run simultaneously, and were separately eluted. Each of the three fractions thus obtained was treated with N-sodium hydroxide solution for 10 min. and, after recovery from the alkaline solutions in the usual way, these were rechromatographed on paper. The alkali treatment resulted in no change in mobility of the 16-oxo-oestradiol-17β-like material, a partial conversion of the 16α-hydroxyoestrone-like material and a complete conversion of the 16β-hydroxyoestrone-like material into products which had the same mobilities as 16-oxo-oestradiol-17β. These findings strongly suggested that the spots detected by paper chromatography in the extract were indeed due to 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β. However, because of the non-specificity of the Folin & Ciocalteu (1927) reagent used for detecting the spots on the paper chromatograms it was deemed necessary to attempt the isolation of the three α-ketols so that they could be characterized by orthodox methods.

Isolation of 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β from pregnancy-urine extracts

The procedure used for the isolation of the α-ketols was a scaled-up modification of that used for the detection of the compounds. The ketonic neutral-phenolic fraction from 200 l. of pregnancy urine was chromatographed on Celite columns. The fractions containing the α-ketols were chromatographed on a large number of sheets of paper. The three fractions obtained by eluting the appropriate areas of the papers, and which were believed to contain respectively the three α-ketols, were each purified by further paper chromatography and the final products acetylated.

The three acetates after purification were identified with certainty as 16α-hydroxyoestrone di-

acetate, 16β-hydroxyoestrone diacetate and 16-oxo-oestradiol-17β diacetate by their melting points, by mixed melting points with authentic specimens of the acetates and by their infrared spectra. The yields of the chromatographically purified products before acetylation were respectively 20·6, 6·2 and 13·1 mg.

EXPERIMENTAL

Methods and Materials

Paper-chromatographic methods. Strips of Whatman no. 42 paper 34 cm. × 12·5 cm. were extracted in a Soxhlet apparatus for 3 days with a mixture of methanol and chloroform. After drying they were immersed in a 2:1 (v/v) methanol–formamide mixture, blotted with filter paper between two sheets of plate glass and dried in a horizontal position at 37° for 45 min.

Analytical chromatograms were carried out by applying to the paper methanolic solutions of the extracts or of the pure compounds in amounts containing about 10–25 μg. of Kober chromogen. After equilibration in the tanks for at least 1 hr. at 18 ± 2° the chromatograms were developed with chloroform for 10–12 hr. The spots were located by spraying the papers with diluted Folin–Ciocalteu phenol reagent and then hanging the papers in a tank over ammonia solution. After a 12 hr. run the distances from the starting line of the 16α-hydroxyoestrone, 16-oxo-oestradiol-17β and 16β-hydroxyoestrone spots were respectively about 26, 23 and 19 cm.

Preparative-scale chromatograms were carried out by applying across the whole width of each paper about 15 mg. of a urinary fraction in methanolic solution in a streak about 1 cm. wide. After development of the chromatogram, strips 1 cm. in width were cut from each side of each paper and the positions of the oestrogen derivatives located on these strips by the Folin–Ciocalteu reagent. The appropriate areas were then cut out from the main part of each paper, and these thoroughly extracted with methanol at room temperature. After evaporation of the methanolic extracts formamide was removed from the eluted fractions by distribution between ether and water; the ether extracts were dried over sodium sulphate and evaporated to dryness.

Melting points. The melting points of the acetates of the α-ketols were determined with a microscope hot-stage, while those of the α-ketols themselves were determined in sealed evacuated capillaries. All melting points were determined with the same thermometer and the values given are uncorrected for emergent stem.

Reference compounds. 16α-Hydroxyoestrone, 16α-hydroxyoestrone diacetate, 16-oxo-oestradiol-17β and 16-oxo-oestradiol-17β diacetate were prepared as described previously (Marrian, Watson & Panattoni, 1957; Marrian, Loke, Watson & Panattoni, 1957). 16β-Hydroxyoestrone diacetate, m.p. 139–141°, $[\alpha]_D^{15}$ + 132° in ethanol (*c*, 0·494), was prepared by our colleague Mr K. H. Loke by a method based on that of Biggerstaff & Gallagher (1957). Hydrolysis of this by the method used for the hydrolysis of 16α-hydroxyoestrone diacetate yielded 16β-hydroxyoestrone, m.p. 237–239° (change of form 232°). As judged by column and paper chromatography this preparation was contaminated with about 5% of 16-oxo-oestradiol-17β.

Isolation of 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β from the urine of pregnant women

Late-pregnancy urine was hydrolysed enzymically in batches of about 16 l. as previously described (Marrian, Watson & Panattoni, 1957). Before its extraction once with an equal volume of ether, Bradosol (a 5% solution of β-phenoxyethyldimethyldodecylammonium bromide; Ciba Laboratories Ltd.) (1 ml./l.) was added to facilitate the breaking of emulsions. The ether extract was washed four times with 5% (w/v) $NaHCO_3$ solution and twice with water, dried over Na_2SO_4 and evaporated to dryness. The neutral-phenolic material from each batch was treated overnight at room temperature with 4 g. of trimethylammoniumhydrazide chloride in 40 ml. of ethanol and 8 ml. of acetic acid, and the ketonic fraction separated in the usual way.

Combined ketonic fractions corresponding to about 50 l. of urine were each chromatographed in a 2 cm. diameter column on 120 g. of Celite with the system 70% (v/v) methanol in water–20% (v/v) hexane in benzene. The first 240 ml. of eluate was rejected and the next 800 ml. containing the α-ketolic oestrogen derivatives was evaporated to dryness. The total weight of chromatographically concentrated material obtained from 200 l. of urine was 1·23 g.

This material was chromatographed on 80 sheets of paper in the formamide/chloroform system. After this purification the crude 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β fractions weighed respectively 121·2, 21·7 and 44·0 mg.

The first of these fractions was rechromatographed on eight sheets of paper, after which 20·6 mg. of material was obtained. The second fraction after rechromatography on two sheets of paper yielded 6·2 mg., while the third after rechromatography on three sheets yielded 13·1 mg. All three fractions were semicrystalline after rechromatography, but the second (16β-hydroxyoestrone) was heavily contaminated by a yellow oil.

The 16α-hydroxyoestrone fraction was acetylated overnight at room temperature with acetic anhydride and pyridine. After one crystallization from ethyl acetate–hexane the acetate melted at 169–172° and had $[\alpha]_D^{16}$ + 141° in ethanol (*c*, 0·508). After admixture with authentic 16α-hydroxyoestrone diacetate (m.p. 171–172°, $[\alpha]_D^{12}$ + 149°) the m.p. was 171–172·5°. The infrared spectrum of the acetate in carbon disulphide solution was identical with that of the authentic sample.

The 16β-hydroxyoestrone fraction after acetylation was still heavily contaminated with a yellow oil, and attempts to crystallize it from ethyl acetate–hexane and methanol were unsuccessful. It was, therefore, chromatographed on a column of 5 g. of aluminium oxide [acid-washed; activated at 120°; deactivated with 5% (v/w) water] with 50% (v/v) hexane in benzene and benzene as eluents. The benzene eluate yielded 4·1 mg. of a gum which yielded 2·1 mg. of white crystals (m.p. 138–141°) on crystallization from methanol. After admixture with authentic 16β-hydroxyoestrone diacetate (m.p. 139–141°) the m.p. was 139–141°. The infrared spectra of the two acetates were identical.

The 16-oxo-oestradiol-17β fraction was acetylated, and after crystallization from ethyl acetate–hexane yielded

7·1 mg. of white crystals which had m.p. 133·5–135·5° and $[\alpha]_D^{18} - 71°$ in ethanol (*c*, 0·536). The mixed m.p. with authentic 16-oxo-oestradiol-17β diacetate [(m.p. 132·5–134°; $[\alpha]_D^{17} - 64°$ in ethanol (*c*, 0·546)] was 130·5–133·5°. The infrared spectra of the two acetates were identical.

DISCUSSION

The isolation of 16β-hydroxyoestrone from the urine of pregnant women described here is the first reported isolation of this oestrogen derivative from a natural source. In view of the ease with which this compound undergoes rearrangement to 16-oxo-oestradiol-17β it seems unlikely that the isolated material was an artifact.

This isolation provides further support for the speculative scheme for the biogenesis of oestriol and 16-*epi*oestriol advanced by Marrian, Watson & Panattoni (1957) and Marrian, Loke, Watson & Panattoni (1957). The two hypothetical intermediates between oestrone and oestriol and 16-*epi*oestriol have now both been shown to be present in pregnancy urine, and it has previously been shown (Brown & Marrian, 1957) that 16α-hydroxyoestrone undergoes metabolic reduction to oestriol in man. It now remains to be shown whether oestrone can undergo 16-hydroxylation to form 16α-hydroxyoestrone and 16β-hydroxyoestrone, and whether the latter can give rise to 16-*epi*oestriol by metabolic reduction.

The isolation of 16-oxo-oestradiol-17β from pregnancy urine by a process which, as far as could be determined, could not have led to the artifactual formation of the compound from either 16α-hydroxyoestrone or 16β-hydroxyoestrone shows that Marrian, Loke, Watson & Panattoni (1957) were incorrect in their view that pregnancy-urine extracts contain no 16-oxo-oestradiol-17β other than that which is formed as an artifact during the isolation process.

As far as can be judged from the yields of the compounds isolated in the course of the present work, 16-oxo-oestradiol-17β must be an oestrogen metabolite quantitatively comparable with 16α-hydroxyoestrone. It is therefore of some importance to consider the metabolic relationships of 16-oxo-oestradiol-17β to the other ring-D-disubstituted oestrogen metabolites. Levitz, Spitzer & Twombly (1958) have recently shown that [16-^{14}C]-oestriol administered to human subjects gives rise to radioactive 16-oxo-oestradiol-17β and 16-*epi*oestriol in the urine. These important findings show clearly that oestriol undergoes partial oxidation at C-16 in the body to form 16-oxo-oestradiol-17β, and that the latter can undergo metabolic reduction, with the formation of 16-*epi*oestriol. The present authors nevertheless believe that the 16-*epi*oestriol may also be formed from oestrone via 16β-hydroxyoestrone, although proof that this is so has yet to be obtained.

Note added in proof. Since the submission of this paper Brown, Fishman & Gallagher (1958) have reported the detection of radioactive 16β-hydroxyoestrone by reverse isotope dilution in the urine of subjects treated with [16-^{14}C]oestradiol-17β.

SUMMARY

1. A ketonic neutral-phenolic fraction obtained from enzymically hydrolysed late-pregnancy urine was subjected to partition chromatography and then paper chromatography. By means of the Folin–Ciocalteu reagent spots corresponding in their mobilities to those of 16α-hydroxyoestrone, 16-oxo-oestradiol-17β and 16β-hydroxyoestrone were detected.

2. By using similar methods on a large scale three semicrystalline concentrates believed to contain the three α-ketolic oestrogen derivatives respectively were prepared from 200 l. of late-pregnancy urine.

3. Acetylation of these concentrates yielded three crystalline acetates which were identified as those of 16α-hydroxyoestrone, 16-oxo-oestradiol-17β and 16β-hydroxyoestrone by melting points, mixed melting points and infrared spectrometry.

4. This demonstration of the occurrence of 16β-hydroxyoestrone in urine is compatible with the previously expressed view (Marrian, Watson & Panattoni, 1957; Marrian, Loke, Watson & Panattoni, 1957) that this compound is the intermediate in the metabolic conversion of oestrone into 16-*epi*oestriol.

5. Since the isolation procedure employed was unlikely to have caused artifactual formation of 16-oxo-oestradiol-17β by rearrangement of 16α-hydroxyoestrone or 16β-hydroxyoestrone, this work supports the view of Levitz, Spitzer & Twombly (1956) that 16-oxo-oestradiol-17β is a naturally occurring oestrogen metabolite.

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Phosphate Esters in the Uterus

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Hollmann (1949) observed that more than half the acid-soluble phosphate esters extracted from cow uterus resisted acid hydrolysis. After 10 hr. in N-HCl at 100°, 72·5% of the organic phosphate was still unhydrolysed, and he suggested that the acid-resistant ester was phosphoglyceric acid.

In a preliminary experiment on an extract from rat uteri, 35% of the acid-soluble phosphate resisted N-HCl at 100° for 3 hr., but very little phosphoglyceric acid was detectable by the enzymic method of Slater (1953). Since Awapara, Landua & Fuerst (1950) have demonstrated the presence of phosphorylethanolamine in extracts of rat uterus, it was decided to make a further study of the phosphate esters in the uterus. Extracts were prepared from non-pregnant uteri of a number of mammalian species, and from pregnant and post-partum rabbit uteri. Phosphate analyses were carried out on all the extracts. The extracts from pregnant and post-partum rabbit uteri were also investigated by paper chromatography.

EXPERIMENTAL

Extracts were prepared in three ways.

(1) A method described by Menkes & Csapo (1952) was used. Rats in oestrus were anaesthetized with Nembutal and the uteri dissected out. Four uteri were extracted together. After 20 min. in ice-cold oxygenated saline, they were blotted dry, frozen with liquid nitrogen and powdered in a mortar. The powder was extracted with 10 vol. of 5% (w/v) trichloroacetic acid, and, after being left for 30 min. at 0°, the extract was filtered.

(2) The uterus was removed from a non-pregnant anaesthetized rabbit and, after the internal surface had been gently scraped, the tissue was frozen in liquid nitrogen. It was ground to a powder and extracted with 5% trichloroacetic acid as in method (1). This method was also used for non-pregnant pig and cow uteri which were brought from the slaughter house packed in ice, immediately after the death of the animal. Human uterus, which was obtained immediately after removal at operation, was extracted in this way.

(3) Extracts were prepared from the uteri of rabbits in the fourth week of pregnancy and just post partum, by the method of Dawson (1955*a*). In this method the danger of the liberation of glycerylphosphorylcholine and glycerylphosphorylethanolamine from the tissue phospholipids is diminished by the substitution of boiling water for trichloroacetic acid.

The animals were stunned and the uteri removed and packed in ice. Further steps were carried out in the cold. Remnants of the placenta were gently removed with a blunt scalpel, care being taken to remove as little as possible of the endometrium. After freezing in liquid nitrogen, the uteri were powdered. The powder was transferred to a beaker, extracted with 4 vol. of boiling water and kept in a boiling-water bath for 2 min. Absolute ethanol (6 vol.) was added and the solution was centrifuged. The supernatant was shaken with chloroform to extract the ethanol, and the aqueous layer was recentrifuged and passed through a column of Amberlite IRC 50 H^+ to remove metal ions. It was then shaken with 2 vol. of butan-2-ol to remove any turbidity and centrifuged. The supernatant was neutralized and dried *in vacuo*, the temperature being kept below 50°. The residue was dissolved in a known volume of water. Each rabbit uterus was extracted separately, and two or three extracts were combined before the material was dried *in vacuo*.

One uterus from a pregnant rabbit was extracted in two different ways: with water and with trichloroacetic acid. The frozen powder was divided into two parts, one part extracted with trichloroacetic acid as in method (1) and the other part with boiling water as in method (3).

Phosphoglyceric acid estimation. The enzymic method of Slater (1953) was used to estimate the phosphoglyceric acid in the trichloroacetic acid extracts from non-pregnant pig, rat, cow and human uteri, and in the water extract from pregnant rabbit uterus. Since this method does not distinguish phosphoglyceric acid from adenosine triphosphate and adenosine diphosphate, the trichloroacetic acid extracts were left overnight at room temperature to allow the hydrolysis of the acid-labile phosphates to occur.

Phosphate estimations. Total phosphate was estimated by the method of Allen (1940), or by a modification of the method of Weil-Malherbe & Green (1951) (Needham & Cawkwell, 1956). Inorganic phosphate was estimated by the method of Fiske & Subbarow (1925) or by that of Weil-Malherbe & Green (1951). All the extracts prepared were analysed for total phosphate, inorganic phosphate and for inorganic phosphate produced after heating for 10 min. and for 3 hr. in N-HCl at 100°. The increase in inorganic phosphate after 10 min. gave a value for the easily hydrolysable phosphate. The further increase in inorganic phosphate after heating for 3 hr. was assumed to be due to the partial breakdown of ribose phosphate from the nucleotides. According to LePage (1945), ribose phosphate is 58·7% hydrolysed after heating in N-HCl for 3 hr. at 100° and this figure was used in calculating the total ribose phosphate present in the extracts. The acid-resistant phosphate fraction was the phosphate remaining unhydrolysed after 3 hr. in N-HCl at 100°, when corrected for the breakdown of ribose phosphate.

Ribose estimations. Ribose was estimated by the method of Mejbaum (1939).

Ethanolamine estimations. Ethanolamine was estimated by measuring the ammonia production after periodate oxidation (Artom, 1945). To remove free ammonia from the samples before oxidation, borate buffer, pH 9·6, was added to the neutralized sample in a Markham (1942) distillation apparatus, and the free ammonia distilled off before the periodate reagent was added.

The ethanolamine estimations were carried out on extracts from pregnant and post-partum rabbit uteri before and after acid treatment, after heating in N-HCl for 1 hr. at 100° and after heating in a sealed tube with 6N-HCl at 123° for 24 hr. The short acid treatment liberates ethanolamine from glycerylphosphorylethanolamine, and the long acid treatment liberates ethanolamine from phosphorylethanolamine.

Choline estimations. Choline was estimated by the reineckate-precipitation method of Engel, Salmon & Ackermann (1954) in material eluted from paper chromatograms. The choline periodide-precipitation method of Appleton, La Du, Levy, Steele & Brodie (1953) was used to estimate the choline in extracts from pregnant and post-partum rabbit uteri before acid treatment, after heating for 30 min. in N-HCl at 100° and after heating for 24 hr. in 2N-HCl at 123° in a sealed tube. Glycerylphosphorylcholine is hydrolysed by the short acid treatment to give free choline and glycerol phosphate, whereas phosphorylcholine is hydrolysed by the prolonged acid treatment to give free choline and inorganic phosphate (Dawson, 1955*b*).

Chromatography

Removal of nucleotides. Before chromatography the extracts were freed from nucleotides by adsorption on charcoal (Norit SX Special, washed with N-HCl to remove inorganic phosphate). The charcoal was removed by filtering through a no. 3 sintered-glass filter. Ribose estimations before and after the charcoal treatment showed that 90% of the nucleotides had been removed.

Two-dimensional chromatograms were run on Whatman no. 1 paper as described by Dawson (1955*b*). The first solvent was phenol saturated with 0·1% (w/v) NH_3 soln.; the second solvent was propanol–aq. NH_3 soln.–water (60 ml. of propanol, 30 ml. of aq. NH_3 soln., sp.gr. 0·880, 10 ml. of water). After running, the chromatograms were sprayed first with 0·1% ninhydrin in water-saturated butanol and then with the acid-molybdate solution of Hanes & Isherwood (1949). The phosphate-containing spots were developed by exposure to ultraviolet light.

Preparative chromatograms were also used. A quantity of charcoal-treated extract corresponding to a total P content of 0·2 mg. was applied as a streak to the paper and run for 18 hr. in the phenol solvent. Marker strips were cut from the middle of the paper and sprayed with ninhydrin and acid molybdate. Bands of the unstained paper with R_F values of 0–0·3, 0·3–0·5, 0·5–0·8 and 0·8–1·0 were eluted with water. The eluates were concentrated *in vacuo* and diluted to a known volume.

When the eluates were treated with HCl and then re-chromatographed the acid was removed by repeated distillation *in vacuo*.

Chromatographic markers. Ethanolamine phosphate was prepared by Mr B. R. Slater according to the method of Outhouse (1937). Glycerylphosphorylcholine was a gift from Dr R. M. C. Dawson.

RESULTS

The results of the ribose and phosphate analyses are given in Table 1. The percentage of acid-resistant phosphate in the total phosphate does not vary greatly with the animal and type of extraction used. The two different methods of estimating ribose phosphate, by ribose and by phosphate estimations, gave results in agreement in two cases and within 20% in the other two cases.

Very little phosphoglyceric acid was found in the extracts in which this compound was estimated. The extracts from rat uteri contained 65 μmoles/100 g. wet wt. The extracts from pig, cow, human and pregnant rabbit uteri contained no detectable phosphoglyceric acid.

Chromatography of the charcoal-treated extracts from the rabbit uteri showed the presence of the esters phosphorylethanolamine, phosphorylcholine and glycerylphosphorylcholine. Glycerylphosphorylethanolamine could be estimated in the extracts from pregnant rabbit uteri, and the chromatograms showed a faint spot in the position of this compound, but enough material was not obtained to identify the products of acid hydrolysis. Phosphoglyceric acid was not detected on the chromatograms.

Identification of phosphorylethanolamine

Two-dimensional chromatograms of the charcoal-treated extracts showed a spot in the position of phosphorylethanolamine which was ninhydrin-positive and stained blue with the acid-molybdate spray. Phosphorylethanolamine, which has R_F 0·39 in the phenol solvent (Dawson, 1954), was eluted in the fraction with R_F 0·3–0·5 from the chromatogram of a streak of the charcoal-treated extract. A sample of this fraction was cochromato-

graphed in two dimensions with a synthetic sample of phosphorylethanolamine. Only one ninhydrin-positive, phosphate-containing spot could be seen. A further sample was given the prolonged acid treatment as described and then chromatographed in phenol. A ninhydrin-positive spot near the solvent front with the same R_F as the marker ethanolamine and an inorganic phosphate spot were seen, but there was no spot in the position of phosphorylethanolamine.

Identification of phosphorylcholine and glycerylphosphorylcholine

Two-dimensional chromatograms of the charcoal-treated extracts showed two spots which stained blue with acid molybdate but which were not ninhydrin-positive. Phosphorylcholine has R_F 0·93, glycerylphosphorylcholine R_F 0·88 in the phenol solvent (Dawson, 1955*b*), therefore both compounds would be present in the fraction with R_F 0·8–1·0 eluted from the chromatogram of a streak of the charcoal-treated extracts. When a sample of the eluted fraction was chromatographed in the propanol–aq. NH_3 soln. solvent, two spots which stained blue with the acid-molybdate spray were seen, with R_F values 0·23 and 0·41, corresponding to phosphorylcholine and glycerylphosphorylcholine respectively.

The presence of combined choline in the eluted fraction was shown by reineckate precipitation and by chromatography. After the prolonged acid treatment of a sample, choline could be precipitated as the reineckate. No reineckate precipitate could be obtained from the untreated fraction. A further sample was given the prolonged acid treatment and then chromatographed in phenol. Free

Table 1. *Soluble phosphates extracted from the uterus*

Trichloroacetic acid is indicated by TCA. All values are expressed as μmoles/100 g. wet wt.

Animals	Extraction	Total P	Inorganic P	Easily hydrolysable P	Ribose P	Ribose	Acid-resistant P
Rats (4)	TCA	1450	356	356	219	266	522
Rats (4)	TCA	1420	355	533	—	—	—
Rabbit	TCA	1680	371	500	290	280	515
Rabbit	TCA	1630	355	290	194	232	790
Pig	TCA	1580	680	74	290	280	538
Cow	TCA	1490	623	216	—	—	—
Human	TCA	1480	555	187	238	—	500
Pregnant rabbit	TCA	1470	455	261	232	—	520
	Water	1140	500	100	164	—	410
Pregnant rabbits (3)	Water	1370	635	77	148	—	510
Rabbit (1), 6 hr. post-partum	Water	1400	570	110	155	—	560
Rabbits (2) 24 hr. post-partum	Water	1190	465	91	155	—	485
Rabbits (2) 24 hr. post-partum	Water	1140	452	91	181	—	420

Table 2. *Ethanolamine in extracts of the uterus before and after acid treatment*

All values are expressed as μmoles of ethanolamine/100 g. wet wt.

	Ethanolamine in original solution	Increase in ethanolamine after prolonged acid treatment	Increase in ethanolamine after short acid treatment
Pregnant rabbits (3)	510	296	55
Rabbits (2), 24 hr. post-partum	500	275	0
Rabbits (2), 24 hr. post-partum	394	252	0

Table 3. *Acid-resistant phosphate esters of the uterus*

All values are expressed as μmoles of P/100 g. wet wt. Esters are phosphorylethanolamine (PE), glycerylphosphorylethanolamine (GPE), phosphorylcholine (PC) and glycerylphosphorylcholine (GPC).

	Acid-resistant phosphate	PE	GPE	PC	GPC
Pregnant rabbits (3)	510	296	55	36	135
Rabbit (1), 6 hr. post-partum	560	—	—	26	148
Rabbits (2), 24 hr. post-partum	485	275	0	42	84
Rabbits (2), 24 hr. post-partum	420	252	0	55	161

choline was detected as a spot on the chromatogram with the phosphomolybdate spray of Chargaff, Levine & Green (1948). The untreated material gave a very faint choline spot.

The presence of glycerylphosphorylcholine in the eluted fraction was confirmed by cochromatography with a marker and by the production of glycerol phosphate after a short acid treatment. When the treated sample was chromatographed in two dimensions, a spot in the position of phosphorylcholine was still present, but the glycerylphosphorylcholine spot had disappeared and a phosphate-containing spot in the position of glycerol phosphate had appeared.

Ethanolamine and choline were estimated in the water extracts from rabbit uteri before and after acid treatment, by the methods described. Serine as well as ethanolamine is estimated by the periodate-oxidation method of Artom (1945), and the large value obtained before acid treatment was due to the presence of free serine detected chromatographically in the extracts. No serine phosphate could be detected, so that all increase in ammonia production by periodate oxidation after acid treatment was assumed to be due to the ethanolamine produced from ethanolamine phosphate. It is, however, possible that serine peptides were present in the extracts and that these liberated free serine after acid treatment. Choline compounds other than phosphorylcholine and glycerylphosphorylcholine may also be estimated by the method of choline estimation used. The results for the ethanolamine estimation are given in Table 2. In Table 3 the results are expressed as μmoles of the phosphate compound which liberates ethanolamine or choline on acid treatment.

DISCUSSION

Phosphorylethanolamine was first isolated by Outhouse (1936) from extracts of bovine malignant tumour, and Awapara *et al.* (1950) identified and estimated this compound in extracts from a number of organs of the rat. Dawson (1955*b*) first identified phosphorylcholine in extracts from rat liver under conditions which ensured that the compound was not formed from phospholipids in the course of preparation of the extracts, and van Heyningen & Pirie (1958) have also found it in the lens. Glycerylphosphorylcholine was first isolated from tissue extracts by Schmidt, Hershman & Thannhauser (1945), who extracted it from incubated ox pancreas, and it was identified and estimated in water extracts from fresh liver by Dawson (1955*b*).

These compounds are known to be important in the metabolism of phospholipids, and this subject has been reviewed by Dawson (1957). Kornberg & Pricer (1952) found that phosphorylcholine was incorporated intact into phosphatidylcholine in rat liver, and it was later found by Kennedy & Weiss (1955) that the nucleotide cytidine triphosphate was required for the incorporation, by the intermediate formation of cytidine diphosphate choline. Phosphorylethanolamine reacts in a similar way to form phosphatidylethanolamine. Phosphorylcholine and phosphorylethanolamine are therefore intermediates in the synthesis of phospholipids. On the other hand, isotopic evidence suggests that glycerylphosphorylcholine and glycerylphosphorylethanolamine are breakdown products of the phospholipids and are not concerned in their synthesis. Dawson (1955*a*) measured the rate of labelling of glycerylphosphorylcholine and glycerylphosphorylethanolamine with ^{32}P in intact rat liver, and compared it with the rate of labelling of the phospholipids. The experiments showed that phosphatidylethanolamine and phosphatidylcholine could be the precursors of the free glycerylphosphorylethanolamine and glycerylphosphorylcholine extracted from the liver. The acid-resistant compounds identified in the rabbit uterus may be similarly related to the metabolism of phospholipids.

Since glycerylphosphorylcholine and glycerylphosphorylethanolamine are breakdown products of phospholipids, it seemed likely that the post-partum uterus would contain more of these compounds than the pregnant uterus. Dawson (1955*c*) found that rat liver contained more glycerylphosphorylcholine and ethanolamine after starvation of the animal for 24 hr., which is known to decrease the total phospolipid of the liver. However, there do not appear to be large differences between the concentration of these compounds in pregnant and post-partum rabbit uteri.

The results of the phosphoglyceric acid estimations are not in agreement with Hollmann's (1949) suggestion that extracts from cow uterus contain a large quantity of phosphoglyceric acid. Purkayastha & Roy (1956), who fractionated the phosphate compounds from extracts of human uterus by the barium-precipitation method, found large quantities of phosphoglyceric acid, but this could not be detected by Slater's (1953) enzymic method.

SUMMARY

1. The phosphate esters in extracts from human, cow, pig and rabbit uteri have been investigated. About 35% of the total soluble phosphate is resistant to acid treatment for 3 hr. in N-HCl at 100°.

2. An extract from rat uteri contained a small quantity of phosphoglyceric acid, but extracts from human, cow, pig and rabbit uteri contained no phosphoglyceric acid.

3. Phosphorylcholine, phosphorylethanolamine and glycerylphosphorylcholine have been identified and estimated in the acid-resistant fraction of extracts from pregnant and post-partum rabbit uteri. Glycerylphosphorylethanolamine was found in the pregnant-rabbit uterus.

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The Reaction of Peptides and Insulin with Methylhydrazine

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The hydrazine method for the determination of the *C*-terminal amino acids of proteins (Akabori, Ohno & Narita, 1952; Ohno, 1953) has been modified by Bradbury (1956) to give improved recoveries of most of the amino acids. Subsequent kinetic studies showed that the increased selectivity of fission of peptide bonds possible in hydrazinolysis, as against acid hydrolysis, is due to the greater importance of steric factors in hydrazinolysis (Bradbury, 1958*a*). In an attempt to increase still further this selectivity the larger molecule methylhydrazine has been reacted with glycylglycine and DL-leucylglycine. The possibilities of the use of methylhydrazine in the determination of the *C*-terminal amino acids of proteins and as a protein solvent have been examined.

EXPERIMENTAL AND RESULTS

Materials

Glycylglycine, DL-leucylglycine and trypsin (Light and Co., Colnbrook, Bucks), insulin (Boots Pure Drug Co., Ltd., Nottingham) and lysozyme (Armour and Co., Chicago) were used without further purification. Merino 64's wool was purified as described previously (Bradbury, 1958*b*). Anhydrous hydrazine was obtained from the hydrate by drying over CaO and distilling (Bradbury, 1958*a*).

Methylhydrazine sulphate (Fisons Chemicals, Wigmore Street, London, W. 1) and a 100% molar excess of solid A.R. NaOH were placed in a flask fitted with a reflux condenser. Sufficient water was added to dissolve the NaOH and the flask cooled to slow the vigorous reaction. The mixture was then distilled under reduced pressure, two fractions being collected, the first of which was rich in

methylhydrazine. This was dried by the addition of freshly ignited CaO, heating at 100° for 3 hr. and then distilling under reduced pressure (10^{-4} mm. Hg) at room temperature. A second drying over CaO gave a product containing 93·5% of methylhydrazine as measured by titration with iodate (Penneman & Audrieth, 1948). This product was used without further purification because it was found previously that 95% hydrazine gave the same rate constants as the anhydrous materials (Bradbury, 1958*a*).

Ethylhydrazine sulphate (Fisons Chemicals, Wigmore Street, London, W. 1) had an apparent purity of 103·5% on titration with iodate. On conversion into ethylhydrazine by the above method an apparent purity of 104·2% was obtained. The product was used without further purification, as it seemed likely that these high results were due to the presence of a small amount of a substituted hydrazine of lower equivalent weight than that of ethylhydrazine.

Procedure and results of rate measurements

The experimental procedure for the determination of the rate of the uncatalysed and acid-catalysed reaction of methylhydrazine with glycylglycine and DL-leucylglycine was the same as that used previously (Bradbury, 1958*a*). The rate of reaction of each dipeptide with methylhydrazine was considerably slower than with hydrazine, necessitating much longer times of reaction at 60° (up to 3 days). In spite of this the recoveries of amino acid and unchanged peptide were comparable with those obtained with hydrazine (Bradbury, 1958*a*). First-order rate constants were obtained graphically for the uncatalysed and the sum of the catalysed and uncatalysed reactions. They were used to calculate the second-order rate constants for the uncatalysed reactions, k''_u, and catalysed reactions, k''_c, as before (Bradbury, 1958*a*). The kinetic scheme proposed in that paper may be incorrect because of the possible similarity of the mechanisms of acid hydrolysis (Martin, 1957) and acid-catalysed hydrazinolysis of peptides; but until this point can be clarified it is valid to continue to assume the present mechanism, provided that no conclusions are based thereon. The rate constants for the reaction of the two dipeptides with methylhydrazine are given in Table 1.

Two experiments made with DL-leucylglycine at 80° and 100° indicate that the temperature-dependence of the rate constants is of the same order as that observed with hydrazine. Treatment of the two peptides with ethylhydrazine gave very low yields of amino acid and unchanged peptide. The reason for this is obscure.

Treatment of insulin with methylhydrazine containing hydrazine sulphate

The method used was that already described for the determination of the *C*-terminal amino acids of proteins by hydrazinolysis (Bradbury, 1958*b*). Insulin was treated with methylhydrazine containing M-hydrazine sulphate (used instead of methylhydrazine sulphate to increase the rate of the reaction) at 60° and 100° for 16 hr. The yields of the *C*-terminal amino acid alanine were 1·7 and 5·4% respectively of the theoretical amount, as compared with 36% (uncorrected) for hydrazine. Also present were the non-*C*-terminal amino acids glycine, serine and glutamic acid in comparable amounts with that obtained previously with hydrazine (see Table 1, Bradbury, 1958*b*).

Hydrazine and methylhydrazine as solvents for proteins

Samples of insulin, trypsin, lysozyme and wool (2 mg. each) were placed in tubes on a vacuum line and 0·2 ml. of anhydrous hydrazine or methylhydrazine was distilled into each. The tubes were sealed off and shaken at 25°, when it was found that both insulin and trypsin dissolved rapidly in both hydrazine and methylhydrazine, producing 1% solutions, but lysozyme and wool were not dissolved by either solvent even after prolonged shaking.

DISCUSSION

The range of reactivities of different peptide bonds towards hydrazine is much greater than towards aqueous hydrochloric acid, owing largely to the operation of steric factors (Bradbury, 1958*a*). On the basis of the so-called intuitive approach to steric hindrance of reactions it would be expected that a further increase of the size of the attacking molecule, in this case the replacement of hydrazine by methylhydrazine, would cause further enhancement of this effect. However, Ingold (1957) quotes several cases of the failure of this intuitive method.

In Table 2 the rate constants are summarized for the reaction of glycylglycine and DL-leucylglycine with hydrazine, methylhydrazine and aqueous hydrochloric acid. From previous work with a wider range of peptides (Bradbury, 1958*a*) it is

Table 1. *Rate constants for the uncatalysed and catalysed reactions of methylhydrazine with glycylglycine and* DL-*leucylglycine at* 60°

The catalysed rate constant was determined from measurements in the presence of M-methylhydrazine sulphate. The density of methylhydrazine used in the calculation of $[CH_3{:}NH{\cdot}NH_2]$ was taken as 0·90 at 60°; an experiment at 20° gave 0·934. $k''_u = k_u/[CH_3{:}NH{\cdot}NH_2]$; $k''_c = k_c/2$; in evaluating k_c it is assumed that $k'_u = k_u$. For further details of symbols, see Bradbury (1958*a*).

Peptide	Uncatalysed rate constant $10^5\,k_u$ (min.$^{-1}$)	Catalysed rate constant $10^5\,(k'_u + k_c)$ (min.$^{-1}$)	$10^5\,k''_u$ (l.mole^{-1} min.$^{-1}$)	$10^5\,k_c$ (min.$^{-1}$)	$10^5\,k''_c$ (l.mole^{-1} min.$^{-1}$)
Glycylglycine	41·6	99	2·12	57·4	28·7
DL-Leucylglycine	2·20	6·6	0·112	4·40	2·20

Table 2. *Comparison of second-order rate constants for the reaction of glycylglycine and* DL-*leucylglycine with hydrazine, methylhydrazine and aqueous hydrochloric acid*

The rate constants for the reactions with hydrazine (see Table 7, Bradbury, 1958*a*) and methylhydrazine were obtained at 60°. Harris, Cole & Pon (1956) determined the hydrolytic rate constants in HCl at 99°.

	Uncatalysed rate constants $10^5\ k_u''$ (l.mole^{-1} min.$^{-1}$) in		Catalysed rate constants $10^5\ k_c''$ (l.mole^{-1} min.$^{-1}$) in		Hydrolytic rate constants $10^3\ k''$
Peptide	Hydrazine	Methylhydrazine	Hydrazine	Methylhydrazine	(l.mole^{-1} min.$^{-1}$)
Glycylglycine	41	2·12	3040	28·7	3·5
DL-Leucylglycine	1·81	0·112	145	2·20	0·7

reasonable to suppose that the ratio k''(Gly.Gly)/k''(Leu.Gly) gives an approximate estimate of the relative selectivities of the reagents towards the splitting of peptide bonds. A simple calculation from the results in Table 2 gives a value of the ratio of 5 for aqueous hydrochloric acid and average values of 16 for methylhydrazine and 22 for hydrazine. Therefore the selectivity of the reagents towards peptide bonds is in the order aqueous acid < methylhydrazine < hydrazine.

The lower reactivity of methylhydrazine as compared with hydrazine towards peptide bonds precludes its use as a reagent for the determination of the *C*-terminal amino acids of proteins, as shown by the failure of the two experiments with insulin. However, it may be useful as a solvent for proteins, since it appears to combine the good solvent properties of hydrazine (as shown by the solubility tests with four proteins) with decreased reactivity towards peptide bonds. For physical measurements on proteins it may be possible to use methylhydrazine in place of hydrazine in order to avoid the degradation which accompanies the use of the latter (Rees & Singer, 1956; Bradbury, 1958*a*).

SUMMARY

1. The kinetics of the uncatalysed and acid-catalysed reaction of glycylglycine and DL-leucylglycine with methylhydrazine were examined by techniques recorded in a previous paper (Bradbury, 1958*a*).

2. Contrary to expectations based on an intuitive approach to steric effects it has been found that methylhydrazine is no more selective in its fission of peptide bonds than is hydrazine.

3. The slow fission of peptide bonds by methylhydrazine, as compared with hydrazine, precludes its use for the determination of the *C*-terminal amino acids of proteins, but is advantageous in its application as a protein solvent.

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Fractionation of Proteins of the Microsomes of Rat Liver by Means of a Non-Ionic Detergent

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Labelled amino acids have been shown to be rapidly incorporated into the proteins of the microsome fraction and particularly into those which are closely associated with ribonucleic acid (Allfrey, Daly & Mirsky, 1953; Hultin, 1955; Littlefield, Keller, Gross & Zamecnik, 1955; Simkin & Work, 1957). Little is known of the nature of the microsomal proteins into which this incorporation occurs. Littlefield *et al.* (1955) found that during short periods after the administration of a radioactive amino acid to a rat, proteins of high specific radioactivity remained insoluble after treatment of the microsomes with sodium deoxycholate. Hultin (1955) and Simkin & Work (1957) obtained micro-

somal protein fractions of different specific radioactivities by extractions with inorganic salts at various pH values. Recently it was shown (Cohn & Butler, 1957*a*, *b*) that treatment of the microsome fraction with a non-ionic detergent released about one-half of the proteins into solution without solubilizing any of the ribonucleic acid. In preliminary experiments it was found that 2 min. after the administration of [^{14}C]phenylalanine the proteins remaining insoluble had a higher specific radioactivity than the solubilized proteins. However, 6 min. after the injection the solubilized proteins were more highly labelled than those which remained insoluble.

It was thought that further fractionation would throw some light on the problem whether the easily solubilized proteins are derived directly from proteins which remain associated with ribonucleic acid. The investigation gave evidence that under the conditions used a number of protein fractions of different specific radioactivities become labelled practically simultaneously.

MATERIALS AND METHODS

Animals. Male Wistar albino rats (200–350 g.) bred at this Institute were kept on a mixed diet. They were fasted for 18–20 hr. before death.

Radioactive amino acids. DL-[3-^{14}C]Phenylalanine (2 mc/m-mole) was prepared by Dr V. C. E. Burnop. For confirmatory experiments generally-labelled L-[^{14}C]phenylalanine (L-[G-^{14}C]phenylalanine) (11·7 mc/m-mole) was obtained from the Radiochemical Centre, Amersham, Bucks.

Administration of radioactive amino acids. The amino acid, dissolved in 0·85% NaCl soln., was injected either intraperitoneally or into the right femoral vein. In preliminary experiments administration into the tail vein was attempted. Low and inconsistent results suggested that administration by this route was often only partly successful. Before an intravenous injection the rat was lightly anaesthetized with ether.

Removal of liver. The rat was perfused with 0·25 M-sucrose under anaesthesia induced by pentobarbitone sodium. The liver was then quickly removed, plunged into ice-cold sucrose (0·25 M) and chopped with scissors.

In experiments designed to examine the incorporation of the radioactive amino acid the rat was killed by breaking its neck, and the liver cut out and chopped after having been plunged into ice-cold 0·25 M-sucrose. These operations took 30–45 sec. to complete.

Preparation of microsome fraction

Tissue suspensions. To each gram of liver 3 ml. of 0·25 M-sucrose was added and the whole placed in the ice-cold glass tube of a Potter–Elvehjem-type homogenizer into which fitted a polythene pestle. The difference between the internal diameter of the tube and the diameter of the pestle was 0·30 mm. Twenty up-and-down strokes were carried out while the pestle was driven at 1700 rev./min. Tissue suspensions and all subcellular fractions were maintained at 0–5° throughout.

Centrifugal fractions. The tissue suspension (11·5 ml. into each tube of the no. 40 head, Spinco centrifuge, model L) was centrifuged at 18 000 *g* (average *g*) for 10 min. A volume (6·7 ml.) of the supernatant liquid was withdrawn from each tube. All these samples from one liver were pooled, and 6·5 ml. was pipetted into fresh centrifuge tubes and 0·25M-sucrose (4·5 ml.) added. The microsome fraction was obtained by centrifuging at 105 000 *g* for 45 min. All but approx. 0·1 ml. of this, the final, supernatant liquid was withdrawn, and the inside of the tube was wiped with filter paper.

Solubilization of microsome fraction

Use of Lubrol W. Lubrol W (Imperial Chemical Industries Ltd.), a condensate of cetyl alcohol and polyoxyethylene, was used at a concentration of 0·25, 0·40 or 0·50% in a 0·25 M-sucrose solution. The microsome pellet was dispersed in this solution with the aid of a loosely fitting polythene pestle. Sufficient Lubrol W solution was added to make up the volume to 11 ml. The liquid became clear within about 2 min. Centrifuging at 105 000 *g* for 2 hr. produced a pellet, described below as the Lubrol pellet, which had the same appearance as the microsome pellet. The supernatant liquid, referred to as the Lubrol supernatant liquid, was withdrawn except for about 0·1 ml. next to the Lubrol pellet.

Use of sodium deoxycholate. The microsome pellet or the Lubrol pellet was solubilized by means of sodium deoxycholate (British Drug Houses Ltd.) according to the technique of Littlefield *et al.* (1955). The liquid (11 ml.) was then centrifuged at 105 000 *g* for 2 hr. The pellet was usually pale brown in colour.

Use of sodium perfluoro-octanoate. The Lubrol pellet was suspended in 0·25 M-sucrose. The addition of 0·1 M-sodium perfluoro-octanoate (Minnesota Mining and Manufacturing Co., St Paul 6, Minn., U.S.A.) dissolved in 0·25 M-sucrose, pH 7·2, produced a rapid clarification of the liquid. It was then centrifuged at 105 000 *g* for 2 hr. The pellet so produced was either light brown or pink in colour.

Fractionation of Lubrol supernatant liquid

After the Lubrol supernatant liquid had been withdrawn $MgSO_4,7H_2O$ was added to saturation. Next morning the turbid liquid was centrifuged at 10 000 *g* for 10 min. to give a pad of solid on top of a clear pale-yellow liquid. This liquid was carefully drawn into a syringe fitted with a long needle so as not to disturb the solid, which contained fraction M_S (Fig. 1). The clear liquid was then dialysed (Visking tubing 32/32 in.) against water until free from $MgSO_4$. During dialysis a fine sediment (fraction W_S) appeared which was subsequently separated by centrifuging. The clear supernatant liquid (W_L) was treated with an equal volume of a saturated $(NH_4)_2SO_4$ solution. The light flocculent precipitate (fraction AS_{50}) was collected by centrifuging (22 000 *g* for 45 min.). The supernatant liquid was then saturated with $(NH_4)_2SO_4$. The following day the light flocculent precipitate (fraction AS_{100}) was collected by centrifuging (44 000 *g* for 60 min.). The sediments containing fractions AS_{50} and AS_{100} were each dialysed (Visking tubing 32/32 in.) against water until free from sulphate. During dialysis fractions AS_{50} and AS_{100} redissolved. After dialysis the liquids were freeze-dried.

Analysis of tissue suspensions and subcellular fractions

Nitrogen. A micro-Kjeldahl method was used to determine total nitrogen.

Phosphorus. After the destruction of organic matter with 60% (w/w) $HClO_4$ (A.R.) the phosphorus was determined colorimetrically by the method of Fiske & Subbarow (1925), $HClO_4$ being used instead of H_2SO_4.

Protein. Total protein in each sample was precipitated with 0·4 N-$HClO_4$ and treated as described by Littlefield *et al.* (1955). The Lubrol supernatant liquid was treated with an equal volume of 20% (w/v) trichloroacetic acid (A.R.). If the Lubrol supernatant liquid was frozen solid before the addition of 10% (w/v) trichloroacetic acid, the proteins appeared to be more readily precipitated. Sometimes the Lubrol supernatant liquid was freeze-dried and excess of detergent removed by extraction with acetone. The protein of fraction M_S was also precipitated with trichloroacetic acid. When much Lubrol W was present trichloroacetic acid appeared to precipitate the whole protein fraction more readily than $HClO_4$.

Lipid phosphorus. Phosphorus extracted by ethanol–ether–$CHCl_3$ (2:2:1, v/v) (Littlefield *et al.* 1955) was taken to be the total lipid phosphorus.

Ribonucleic acid. The extinction at 260 mμ of acidified alkaline digests (Littlefield *et al.* 1955) served for the estimation of ribonucleic acid. Analyses for ribonucleic acid phosphate showed that this procedure could satisfactorily apply to all further fractions obtained from the microsome fraction.

Treatment of radioactive protein fractions

Precipitation of proteins. DL- or L-Phenylalanine (3 mM) was included in both $HClO_4$ and N-NaOH solutions to facilitate the washing of adsorbed radioactive material off the precipitate. In one experiment a suspension of rat liver was prepared in the presence of DL-[3-^{14}C]phenylalanine (0·5μc/g. of fresh liver). The proteins of the microsome fraction and of the final supernatant liquid were precipitated and isolated as described above. The radioactivity recovered with the protein fraction was negligible irrespective of whether or not the microsome pellet was suspended in sucrose before the addition of $HClO_4$ or whether DL-phenylalanine was present in the $HClO_4$ and N-NaOH solutions. However, protein fractions were isolated in the presence of DL- or L-phenylalanine as a routine.

Isolation of protein fractions soluble in magnesium sulphate. Fraction W_S was left in the centrifuge tube and freeze-dried. Samples of fraction W_L, AS_{50} and AS_{100} were also obtained by freeze-drying the respective dialysate.

Radioactive assays. The final protein residue was assayed for radioactivity on polythene planchets (1 or 2 cm.²) in an end-window counter connected to a scaling unit. When the amount of sample was insufficient to give infinite thickness the proportion of the maximum radioactivity assayed was ascertained from a saturation curve. Since samples of the fractions of the Lubrol supernatant liquid contained considerable amounts of non-protein matter, mostly Lubrol, their specific radioactivities were all calculated to the same nitrogen content (14·3%).

RESULTS

Fractionation of microsomal proteins

Solubilization of microsomal proteins with Lubrol W. When the microsome fraction was resuspended in a solution of Lubrol W of a concentration above 0·25% the pellet obtained on centrifuging contained almost all the ribonucleic acid, about one-half of the proteins and between 20 and 40% of the lipid phosphorus of the microsome pellet (Cohn & Butler, 1957*a*).

Treatment of Lubrol pellet with sodium perfluoro-octanoate. When sodium perfluoro-octanoate was added to a Lubrol pellet resuspended in 0·25 M-sucrose soln. the pellet obtained after recentrifuging contained 80–90% of the ribonucleic acid, 20–30% of the protein and 25% of the lipid phosphorus of the microsome fraction, if the final concentration of sodium perfluoro-octanoate was

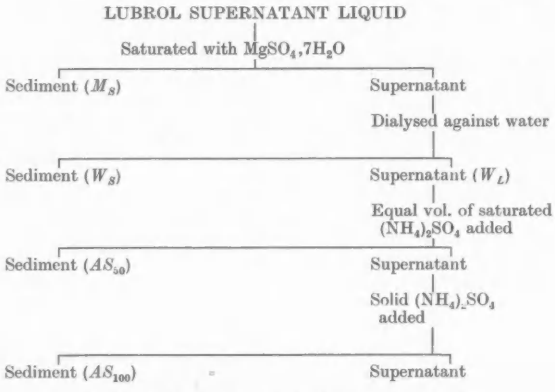

Fig. 1. Fractionation of Lubrol supernatant liquid.

Table 1. *Effect of concentration of Lubrol W on the specific radioactivities of proteins of the pellet and supernatant liquid*

DL-[3-^{14}C]Phenylalanine (2 mc/m-mole) was administered intraperitoneally (0·20 mc/kg.) and the liver microsomes were subsequently isolated and partly solubilized with Lubrol.

Expt. no.	Period after injection (min.)	Concn. of Lubrol (%)	Specific radioactivity of proteins (μc/g.) Lubrol pellet	Lubrol supernatant liquid
1	6	0·25	0·78	1·33
		1·0	0·70	1·16
2	31·5	0·25	2·47	5·40
		1·0	2·45	5·12
		1·0	2·40	5·85
3	55	1·0	2·40	5·24
		2·0	2·41	5·36

0·32 % (7·5 mM). These constituents of the perfluoro-octanoate pellet were thus present in the ratios ribonucleic acid:protein:phospholipid (1:2–3:1·5). The mean phosphorus content of the phospholipids was taken to be 4 % (Swanson & Artom, 1950). The amounts of protein and phospholipid in the pellet increased when the concentration of sodium perfluoro-octanoate was lowered. At concentrations above 0·32 % increasing amounts of ribonucleic acid as well as protein and phospholipid remained in the supernatant liquid.

Lubrol supernatant liquid. On saturating the Lubrol supernatant liquid with magnesium sulphate the precipitate that formed (M_S) contained about 20–25 % of the proteins, most of the lipid phosphorus and about half the Lubrol W present in the liquid. The proteins soluble in water constitute 40–50 % of the Lubrol supernatant liquid. The fraction precipitated by half-saturated ammonium sulphate (AS_{50}) forms approximately two-thirds of the water-soluble proteins, the remainder being fraction AS_{100}.

Distribution of specific radioactivities among cell fractions after the administration of [^{14}C]*phenylalanine.* Table 1 gives some representative results to demonstrate the effect of three different concentrations of Lubrol W on microsomal proteins labelled by previous administration of [^{14}C]phenylalanine. These results show that within the range of concentrations 0·25–2·0 % (w/v) the specific radioactivities of the protein of the pellet and the supernatant liquid remain constant. For very short periods of incorporation the proteins of the Lubrol pellet appeared to have a higher specific radioactivity than those of the supernatant liquid (Table 2). If the rat was allowed to live for at least 6 min. after the injection the specific radioactivity of the proteins of the Lubrol supernatant liquid rose to values more than twice as high as those of the pellet.

Table 2 shows the effect of time on the incorporation of [^{14}C]phenylalanine into the proteins of those microsomal fractions insoluble in Lubrol W. The specific radioactivities of the total proteins of the microsome pellet, the Lubrol supernatant liquid and the final supernatant liquid remaining after removal of the microsomes are given for comparison. It can be seen that the specific radioactivities of proteins of both the microsome fraction and the final supernatant liquid rise during the period examined in the manner previously described by Keller, Zamecnik & Loftfield (1954). For short periods of incorporation the specific radioactivity of the protein remaining with the pellet after treatment of the Lubrol pellet with sodium deoxycholate is greatly increased, showing that proteins of low specific radioactivity are removed. The pellet obtained in this way had the highest specific radioactivity of any microsomal protein fraction examined (Expt. 2). The action of sodium perfluoro-octanoate (Expt. 1) was similar, but the proteins of the pellet had a somewhat lower specific radioactivity than with sodium deoxycholate. The sodium perfluoro-octanoate pellet would thus appear to contain some proteins of the Lubrol pellet of relatively low specific radioactivity which are solubilized by sodium deoxycholate. Two minutes after the administration of [^{14}C]phenylalanine the ratio of the specific radioactivity of the protein of the sodium perfluoro-octanoate pellet to that of its supernatant liquid was 4:1. When the period of incorporation was 6 min. (Expt. 4) the proteins of the sodium perfluoro-octanoate pellet were about 2·5 times as radioactive as those of its supernatant liquid. When the rat was killed 10·5 min. after the injection further solubilization of the Lubrol pellet with sodium deoxycholate (Expt. 5) produced two fractions of equal specific radioactivities. The figures for Expt. 7 show that 30·5 min. after the injection of [^{14}C]phenylalanine the specific radioactivity of the proteins of the pellet after treatment with sodium deoxycholate is well below that of the supernatant liquid. The specific radioactivity of the proteins of the sodium perfluoro-octanoate pellet, however, can be seen to be slightly above that of the proteins of the supernatant liquid, both 10·5 and 30·5 min. after the administration of [^{14}C]phenylalanine. It is obvious that the ratio of the specific radioactivity of the proteins in the pellet and supernatant fractions produced by sodium perfluoro-octanoate falls with time after injection from its maximum to a value close to unity. In contrast, treatment with sodium deoxycholate causes, 30 min. after injection, a drop to 0·61 in the ratio of the specific radioactivity of the proteins in the pellet and supernatant fraction.

Table 2. *Specific radioactivities of proteins of microsomal fractions not solubilized by Lubrol W*

DL-[3-^{14}C]Phenylalanine (2 mc/m-mole) was injected into the right femoral vein (dose was 0·20 mc/kg., except for rat no. 4 which was 0·12 mc/kg.).

			Specific radioactivities of proteins (μc/g.)							
					Lubrol pellet resuspended in					
					Lubrol		Sodium deoxycholate		Sodium perfluoro-octanoate	
Rat no.	Body wt. (g.)	Period (min.)	Final supernatant liquid‡	Microsomes	Pellet	Supernatant liquid	Pellet	Supernatant liquid	Pellet	Supernatant liquid
1	320	2	0·14	0·65	0·77	—	—	—	1·16	0·29
2	350*	2	0·11	0·71	0·90	0·46	1·95	0·39	—	—
3	255	2	0·19	1·03	—	1·00	—	—	1·71	0·28
4	270	6	0·25	1·03	0·56	—	—	—	0·92	0·35
5	230	10·5	0·68	2·22	1·34	2·91	1·43	1·38	—	—
6	320	10·5	0·47	1·85	—	—	—	—	1·08	0·92
7	320†	30·5	0·76	1·94	—	—	0·88	1·45	1·17	0·88

* Injection into tail vein.
† L-[G-^{14}C]Phenylalanine (11·7 mc/m-mole) was injected into the right femoral vein (0·037 mc/kg.).
‡ Final supernatant liquid remaining after removal of microsomes.

Table 3. *Microsomal proteins solubilized and not solubilized by Lubrol W; comparison of radioactivity of fractions*

DL-[3-^{14}C]Phenylalanine (2 mc/m-mole) or L-[G-^{14}C]phenylalanine (11·7 mc/m-mole) was injected into the right femoral vein.

				Relative specific radioactivity of proteins of microsomal fractions (total proteins of microsomes = 100)										
					Lubrol pellet resuspended in									
					Sodium deoxycholate		Sodium perfluoro-octanoate							
Expt.	Radioactivity of injected phenyl-alanine (μc/kg.)	Period (min.)	Specific radio-activity of microsomes (μc/g.)	Lubrol pellet	Pellet	Supernatant liquid	Pellet	Supernatant liquid	M_S	W_S	W_L	AS_{50}	AS_{100}	Rat no. in Table 2
A	200 (DL)	2	0·65	118	—	—	179	45	37	91	148	65	202	1
B	33 (L)	2	0·63	83	—	—	129	33	82	87	148	83	—*	—
C	120 (DL)	6	1·03	54	—	—	89	34	106	108	204	135	344	4
D	45 (DL)	6	0·62	—	—	—	65	42	123	126	202	140	229	—
E	10 (L)	6	0·11	86	—	—	119	43	133	—*	114	100	354	—
F	100 (L)	6	2·84	58	—	—	65	37	116	—*	161	165	252	—
G	200 (DL)	10·5	1·85	—	—	—	58	50	62	120	207	—	—	6
H	37 (L)	30·5	1·94	—	45	75	60	45	105	—*	—	241	402	7

* Sample was insufficient for radioactive assay.

The results of the salt fractionation (Fig. 1) of labelled proteins of the Lubrol supernatant liquid are shown in Table 3. The values for the radioactivities of all the fractions are expressed as specific radioactivities relative to those of the total microsome proteins (= 100), so that the results of experiments with different doses of [^{14}C]phenylalanine may be compared. The specific radioactivity of fraction W_S is appreciably less than that of fraction W_L. Fraction AS_{50} has a lower specific radioactivity than fraction AS_{100}. This final fraction AS_{100}, which has the highest specific radioactivity of any fraction in the Lubrol supernatant liquid, can be seen to be slightly more radioactive than the proteins of the sodium perfluoro-octanoate pellet even 2 min. after the administration of [^{14}C]phenylalanine (Expt. *A*). On the whole the same pattern of distribution of radioactivity is shown at all the times examined. The behaviour of fraction M_S is somewhat variable and no regularity has been noticed. When the specific radioactivities of these protein fractions of the Lubrol supernatant liquid are compared with those of the Lubrol pellet the figures show that, with the exception of the experiments at 2 min. and also Expt. *E*, which is anomalous, the Lubrol pellet and its fractions have a lower radioactivity than the fractions of the Lubrol supernatant liquid. The results in Table 3 show that whereas the relative specific radioactivities of the Lubrol and the sodium perfluoro-octanoate pellets decreased with the length of the period of incorporation, the relative specific radioactivities of the fractions of the Lubrol supernatant liquid give evidence of a rise taking place between 2 and 6 min. after injection.

DISCUSSION

The present investigation has given further evidence of the existence of different microsomal protein fractions distinguishable by the extent of incorporation of a labelled amino acid. The first step in the process of fractionation, the treatment with a non-ionic detergent to produce a soluble protein fraction almost free from ribonucleic acid, may be compared with the solubilization of microsomal proteins which Littlefield *et al.* (1955) achieved by means of sodium deoxycholate. In the present investigation Lubrol W solubilized about one-half by weight of microsomal protein as compared with five-sixths which was solubilized by sodium deoxycholate. However, the protein solubilized by Lubrol W was more highly labelled than the larger quantity of protein solubilized by sodium deoxycholate, showing the presence of a fraction of low radioactivity in the sodium deoxycholate solution.

There is so far no evidence to suggest if and how such fractions as AS_{100} or its constituent proteins are derived from possible precursors in ribonucleoproteins. It was noteworthy, however, that the specific radioactivities of fractions M_S, W_S, AS_{50} and AS_{100} all rose at approximately the same rate as the specific radioactivity of total microsomal proteins. This simultaneous labelling of proteins should be contrasted with the results of Simkin & Work (1957), who found that the pattern of the specific radioactivities of all their protein fractions changed with time over a period of 3 hr. after injection of labelled amino acids. Although no information is available at present on the morphological side of this problem, the presence of ribonucleic acid in all the fractions examined by Simkin & Work (1957) would suggest that sodium chloride at different pH values breaks up microsomal protein and nucleoprotein complexes in a manner different from that of solubilizing agents. Since guinea pigs were used by Simkin & Work and rats in the present work a difference in behaviour of microsomes between these species cannot be ruled out.

The chemical nature of the different fractions of the present work has not been investigated further, but the solubility in half-saturated ammonium sulphate solution of the final fraction (AS_{100}) suggests that it may be an albumin, and it would be interesting to discover if it is related to serum albumin. In this connexion it may be significant that serum albumin having a high specific radioactivity has recently been detected by an immunological technique in liver microsomes (Campbell, Greengard & Kernot, 1958).

SUMMARY

1. The microsome fraction from rat liver was treated with the non-ionic detergent Lubrol W, which solubilized approximately one-half of the protein, 60–80% of the lipid phosphorus and a negligible amount of ribonucleic acid.

2. When rats were killed 2 min. after the injection of [^{14}C]phenylalanine a higher specific radioactivity was found in the protein of the Lubrol pellet than of the supernatant liquid. Six minutes or longer after injection the specific radioactivity of the protein of the Lubrol supernatant liquid was more than twice that of the protein of the pellet.

3. Treatment of the Lubrol pellet with either sodium perfluoro-octanoate or sodium deoxycholate released proteins which, 2 min. after injection, possessed a low specific radioactivity. The pellets so obtained had a higher specific radioactivity than almost all other microsomal protein fractions examined.

4. The proteins of the Lubrol supernatant liquid were separated into four fractions, differences in solubility in strong solutions of magnesium sulphate, ammonium sulphate and water being utilized. The specific radioactivities of these fractions showed the same pattern of distribution, whatever the time interval after the injection of [^{14}C]phenylalanine. The final fraction containing these proteins least readily precipitated became labelled very quickly; 2 min. after injection it had a specific radioactivity as high as that of the proteins of the sodium perfluoro-octanoate pellet.

5. The present results indicate that a number of microsomal proteins which can easily be solubilized become labelled simultaneously.

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Preparation of some Deoxyribonucleic acid–Protein Complexes from Rat-Liver Homogenates

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Preparation of deoxyribonucleic acid from rat-liver homogenates by the action of various salts and phenol (Kirby, 1957) showed that deoxyribonucleic acid free from protein could be isolated when the salt was *p*-aminosalicylate but that about 1% of residual protein remained attached to the deoxyribonucleic acid when benzoate was used. These results suggested that some deoxyribonucleic acid may be bound to the protein through metal linkages, and it was thought that further information could be obtained by isolation and analysis of deoxyribonucleic acid produced by the action of different salts.

For this investigation fluoride and azide were chosen as typical inorganic salts with considerable power of complexing with metals. The action of diethyldithiocarbamate was compared with that of diethylacetate and the action of 2-nitroso-1-naphthol-4-sulphonate with 1-naphthol-4-sulphonate, since the first and third of this group have considerable chelating abilities. *cyclo*Hexanediamine-*NNN'N'*-tetra-acetate was examined because this agent was effective in releasing deoxyribonucleic acid from rat liver whereas a very similar chelating agent, ethylenediamine-*NNN'N'*-tetra-acetate, was not (Kirby, 1957). Finally the effect of benzoate at different concentrations was investigated to determine the effect of ionic concentration on the amount of residual protein attached to the deoxyribonucleic acid. It had been demonstrated previously that deoxyribonucleic acid extracted in the presence of 0·15 M-salts contained more protein than when 0·3 M-salts were used.

MATERIALS AND METHODS

Sodium salts were used in all cases and adjusted to pH 6·5–7·0 with NaOH or acetic acid with narrow-range indicator papers or a pH meter.

Isolation procedure. This was essentially as described previously. The animals were killed and their livers excised and dropped on to solid CO_2. The livers (usually 50–75 g.) were broken up in the appropriate salt solution (400–600 ml.) in a Waring Blendor for about 45 sec. The mixture was stirred and an equal volume of 90% phenol (w/w) was added immediately and stirring continued for 40 min. The conditions of centrifuging depended upon the viscosity of the aqueous layer, which was much more viscous when

more than about 5% of protein was present with the deoxyribonucleic acid (DNA). In these cases the mixture was centrifuged for 1 hr. at 0° at 700 *g*, the dark phenol layer removed by suction and the aqueous layer was centrifuged again in a Servall machine in polyethylene tubes for 1 hr. at 0° at 10 000 *g*. When the high viscosity precluded suction the aqueous solution was separated by careful pouring. When the salt concentration was less than 0·15 M the aqueous solution was made 4% with respect to sodium acetate before addition of an equal volume of 2-ethoxyethanol for precipitation.

DNA with only traces of protein which was precipitated for the first time with 2-ethoxyethanol was more difficult to collect on a rod or a spatula than DNA with more protein, and was better separated by centrifuging for about 1 min. at 500 *g*.

After further precipitations and treatment with ribonuclease the DNA–protein complex was separated from residual glycogen by extraction from 1·25 M-phosphate solution with 2-methoxyethanol, when the complex was extracted entirely into the organic layer and the glycogen remained partially insoluble and partially in the aqueous layer. After centrifuging and dialysis of the organic layer sodium acetate was added and the DNA material was precipitated by 2-ethoxyethanol. The white fibres were washed twice with ethanol–water (3:1), and allowed to stand for several hours over ethanol and then dried in a vacuum desiccator over $CaCl_2$.

Analyses. Determinations of N and P were carried out on materials containing 10–15% of moisture. $\epsilon_{(P)}$ values (Chargaff, 1955) were determined in 0·1 M-NaCl soln. When little protein was present the samples dissolved easily on shaking, but when much protein was present it was sometimes necessary to dissolve the product in water and then add an equal volume of 0·2 M-NaCl soln. Base compositions were estimated as described by Kirby (1957).

Amino acid determination. This was carried out essentially by the method used before (Kirby, 1957). The complex (15 mg.) was hydrolysed with 5·8 N-HCl, and the HCl evaporated, and the residue evaporated two or three times in the presence of acetic acid–water (1:4) and then dissolved in 0·1 ml. of acetic acid–water (1:4); 0·02 ml. was used for separation by paper chromatography and electrophoresis. A sample (7–10 mg.) of the product from the 0·07 and 0·14 M-benzoate preparation was used for hydrolysis, and the residue was dissolved in 0·2 ml. of acetic acid–water (1:4) and 0·01 ml. was used for each separation. Lysine and arginine were determined on Whatman no. 4 paper by single-way chromatography. After the separation colours were developed by spraying the papers with a solution of ninhydrin, and the colours were stabilized by spraying with a solution of cupric acetate monohydrate (150 mg., powdered in 6 ml. of water and 94 ml. of ethanol). The colours were eluted for 2 hr. with 80% methanol (80% 2-methoxyethanol has been found to be better than 80% methanol) and measured at 500 mμ.

Action of p-aminosalicylate and phenol on the deoxyribonucleic acid prepared by the action of 0·07 M-benzoate. DNA prepared by the action of 0·07 M-benzoate (54 mg.) was dissolved in water (100 ml.). To this solution sodium *p*-aminosalicylate (6 g.) and 90% phenol (w/w) were added and the mixture was stirred for 40 min. at room temperature. The mixture was centrifuged at 0° in an International centrifuge at 700 *g* for 45 min. The top layer was siphoned off and the DNA was precipitated with an equal volume of 2-ethoxyethanol. The fibrous precipitate was collected, and dissolved immediately in water (50 ml.); sodium acetate (2 g.) was added and the material was again precipitated with 2-ethoxyethanol (50 ml.). The insoluble product was removed, washed twice with 75% ethanol and allowed to stand for 16 hr. with absolute ethanol before pouring away the ethanol and drying the residue in a vacuum desiccator over $CaCl_2$. The yield was 22 mg.

Action of sodium benzoate and sodium azide on rat-liver homogenates. Rat liver (32 g.) was homogenized in a solution (250 ml.) of sodium benzoate (0·28 M) and sodium azide (0·03 M). An equal volume of 90% phenol was added and the mixture was stirred for 40 min. and then centrifuged for 1 hr. at 0° at 700 *g*. The lower phenol layer was removed and the top layer was centrifuged again. The clear supernatant was removed, and the DNA was precipitated with an equal volume of 2-ethoxyethanol and separated by centrifuging for 1 min. at 700 *g*. The DNA dissolved quickly in water and the separation procedure followed the method described previously (Kirby, 1957). The yield was 56 mg.

Isolation of deoxyribonucleic acid from rat-liver homogenate by the action of ethylenediamine-NNN′N′-tetra-acetate, naphthalene-2-sulphonate and phenol. Sodium naphthalene-2-sulphonate (17·5 g.) and tetrasodium ethylenediamine-*NNN′N′*-tetra-acetate (5 g.) were dissolved in water (500 ml.) and acetic acid (0·5 ml.) was added to bring the pH to 6·5. This solution (200 ml.) was used to homogenize 25 g. of rat liver and the mixture was stirred for 40 min. with 90% phenol (w/w) (200 ml.), after which DNA was isolated exactly as described previously. The yield was 41 mg.

Isolation of deoxyribonucleic acid–protein complex in the presence of free deoxyribonucleic acid. DNA (41·8 mg.) in which no protein was present was dissolved in water (250 ml.), sodium benzoate (5 g.) was added and this solution was used to homogenize 5 g. of rat liver. The homogenate was stirred with 250 ml. of 90% (w/w) phenol for 40 min. and the DNA was isolated exactly as described previously. The product had a lower viscosity than that usually isolated by the 0·14 M-benzoate procedure.

Hydroazinolysis. Anhydrous hydrazine was prepared by Braunitzer's (1955) method and the course of the experiments followed from his data. DNA (30 mg. each from 0·07 and 0·14 M-benzoate preparations) was used with 0·3 ml. of anhydrous hydrazine and *p*-nitrobenzaldehyde in ethyl acetate was used to remove the hydrazones. The final aqueous solutions were yellow. They were evaporated to dryness, the residue was dissolved in 0·1 ml. of acetic acid–water (4:1) and 0·02 ml. was used for chromatographic analysis, which was carried out as described by Kirby (1957). Only the DNA prepared by the action of 0·07 M-benzoate developed any colours with ninhydrin and the amino acids found were: leucine, alanine, glycine, aspartic acid, glutamic acid and serine.

RESULTS

Yields. The yield of the DNA–protein complexes isolated by the phenol procedure is usually 160–220 mg./100 g. of rat liver, irrespective of the amount of protein in the complex. Hence less

DNA is actually extracted by 0·07 and 0·14 M-benzoate solutions since about 50 and 30% respectively of the product is protein. There is no alteration in the base composition with varying amounts of protein.

Amino acid analyses. The analytical results of the various preparations are shown in Table 1. Apart from the amino acids reported in the table, cysteine, serine, threonine, glycine, alanine, proline, methionine and valine are present in the samples prepared by the action of 0·07 and 0·14 M-benzoate and 0·3 M-diethylacetate ions. Glycine is, of course, present in all hydrolysates owing to decomposition of the purines. Cysteine and proline are absent from the chromatograms of all other samples and so, presumably, are present in small quantities in the three preparations mentioned. Serine, threonine, alanine, methionine and valine are also present in preparations made with 0·21 M-benzoate, *cyclo*hexanediamine-*NNN'N*-tetra-acetate, 2-nitroso-1-naphthol-4-sulphonate and to a less extent in the azide and fluoride preparations.

Action of naphthalenesulphonic acids. Naphthalene-2-sulphonate was previously reported (Kirby, 1957) to be moderately active in releasing DNA, but only very little DNA could be isolated when the salt was used at 0·15 M concentration, although the solution gave a strong Dische (1930) reaction. 1-Naphthol-4-sulphonate (0·15 M) yielded 150 mg. of DNA/100 g. of rat liver but some ribonucleic acid (RNA) was present. Naphtholsulphonate was originally considered to be moderately effective since the DNA was not fibrous on precipitation. This, however, was due to coprecipitation of 1-naphthol-4-sulphonate; this was separated during the two-phase separation with 2-methoxyethanol when the DNA was extracted into the organic layer, whereas the naphtholsulphonate was largely insoluble. After dialysis of the organic layer the DNA precipitated in a fibrous manner. The presence of RNA may therefore be due to some inactivation of ribonuclease by 1-naphthol-4-sulphonate.

Since a solution of 2-nitroso-1-naphthol-4-sulphonate is saturated at a concentration of 0·03 M the comparative ability of naphthalene-2-sulphonate and 1-naphthol-4-sulphonate to release DNA under the conditions used at this concentration was examined. No DNA was released by naphthalene-2-sulphonate and only sufficient to produce a colour reaction by 1-naphthol-4-sulphonate. A good yield of DNA was produced by 2-nitroso-1-naphthol-4-sulphonate, although the final product contained some protein and was very faintly yellow.

Amino acids present after two treatments or combinations of salts. The analytical figures for the product of the reaction of *p*-aminosalicylate on the

Table 1. *Analytical figures for deoxyribonucleic acid–protein complexes*

Bases are expressed as moles/100 moles of total bases. Amino acids are expressed as a percentage of the DNA–protein complex isolated. $\epsilon_{(P)}$ values were estimated in 0·1 M-NaCl solution.

Salt	...	...	NaF	NaN_3	Sodium benzoate					Sodium diethyldithiocarbamate	Sodium diethylacetate	*cyclo*Hexanediaminetetra-acetate	2-Nitroso-1-naphthol-4-sulphonate
Concn. (M)	...	...	0·3	0·3	0·35	0·28	0·21	0·14	0·07	0·3	0·3	0·15	0·03
Guanine			22·0	21·2	21·3	21·0	21·8	22·1	21·6	21·0	22·6	20·6	20·8
Adenine			30·3	29·5	29·8	29·7	29·7	29·8	30·6	30·2	29·7	29·5	30·2
Cytosine			18·9	19·7	20·3	20·2	19·3	20·0	19·1	20·8	19·1	20·6	19·2
Thymine			28·8	29·6	28·6	29·1	29·3	28·1	28·7	28·0	28·6	29·3	29·8
N			11·8	12·2	11·75	10·7	12·3	11·75	13·0	12·1	12·65	12·0	13·3
P			7·4	7·8	7·2	6·65	6·65	6·0	5·2	7·7	6·4	7·1	7·14
N/P			1·59	1·57	1·64	1·61	1·85	1·95	2·5	1·58	1·98	1·7	1·87
$\epsilon_{(P)}$			—	6250	6620	7500	7650	8600	9250	6100	7600	7050	7100
Lysine			0·65	0·6	0·14	0·38	0·77	7·00	11·7	0·03	1·20	0·37	1·5
Arginine			0·21	0·22	0·10	0·2	0·28	3·00	5·6	0·05	0·55	0·22	0·7
Aspartic acid			0·11	0·13	0·11	0·21	0·19	5·00	10·5	0·10	0·82	0·65	1·0
Glutamic acid			0·13	0·15	0·09	0·2	0·30	4·3	7·0	0·05	0·75	0·45	0·45
Tyrosine			—	0·10	—	—	0·10	1·4	3·3	—	0·25	0·13	—
Phenylalanine			0·10	0·11	—	—	0·20	2·3	4·5		0·43	0·29	
Leucine/isoleucine			0·83	0·57	0·13	0·17	0·57	12·6	16·5		2·9	0·72	2·00

DNA–protein complex produced by the action of 0·07 M-benzoate are shown in Table 2. The values indicate that about two-thirds of the residual protein was removed in this reaction.

The amino acid residues present after the treatments with ethylenediamine-*NNN'N'*-tetra-acetate combined with naphthalene-2-sulphonate and azide combined with benzoate are shown in Table 3.

Action of liver homogenate on protein-free deoxyribonucleic acid. Since it was possible that DNA might interact with cellular proteins during its liberation from the nuclei, an experiment was performed in which DNA free from protein was mixed with a rat-liver homogenate in 0·14 M-benzoate. The mixture was then extracted with phenol and the DNA isolated in the usual manner. If the added DNA absorbed no protein and the DNA from the liver homogenate had about 30 % of protein, which was the amount found when DNA was isolated from liver in the presence of 0·14 M-benzoate, then the total DNA isolated should have about 6 % of protein. Approximately half this value was found to be present and the results are shown in Table 4.

Table 2. *Analysis of some amino acids after reaction of the deoxyribonucleic acid–protein complex produced by* 0·07 M-*benzoate with* p-*aminosalicylate and phenol*

Amino acids are expressed as a percentage of total material.

Lysine	3·3
Arginine	2·9
Aspartic acid	2·9
Glutamic acid	2·5
Tyrosine	1·3

Table 3. *Amino acid analyses of deoxyribonucleic acid produced by actions of naphthalene-2-sulphonate combined with ethylenediaminetetra-acetate* (*a*) *and benzoate combined with azide* (*b*)

Amino acids are expressed as percentage of total weight.

	(*a*)	(*b*)
Lysine	0·23	0·33
Arginine	0·08	0·13
Aspartic acid	0·16	0·16
Glutamic acid	0·16	0·20
Leucine	0·30	—

Table 4. *Analyses of some amino acids present after extraction of rat liver with* 0·14 M-*benzoate and phenol in the presence of deoxyribonucleic acid*

Amino acids are expressed as a percentage of total material.

Lysine	0·60
Arginine	0·31
Aspartic acid	0·40
Glutamic acid	0·36
Phenylalanine	0·20

Physical measurements. DNA samples prepared by the actions of *p*-aminosalicylate, diethylacetate and 0·07 M-benzoate ions have been examined by Dr K. V. Shooter in a Spinco ultracentrifuge fitted with an ultraviolet-light-absorption optical system. For these experiments 0·005 % solutions of DNA in 0·2 M-NaCl were used. The sedimentation coefficient-distribution curves were calculated in the manner described by Shooter & Butler (1956).

The sedimentation coefficient–distribution curves for the DNA prepared with *p*-aminosalicylate covered a range from 10*S* to 60*S*, the average sedimentation coefficient being 24*S*. This distribution was very similar to those observed for DNA from other animal tissues (Shooter & Butler, 1956). DNA from rat liver prepared by the action of 0·3 M-diethylacetate and 0·07 M-benzoate ions had average sedimentation coefficients of 30*S* and 31*S* respectively. The distribution curves indicated that the preparation with about 7·5 % of protein had 5 % of particles with $S > 60$ and when 50 % of protein was present 20 % had $S > 60$. Butler, Phillips & Shooter (1957) have shown that aggregates of DNA cross-linked by protein can be broken down by treatment with chymotrypsin. Treatment of rat-liver DNA with this enzyme showed that the preparation with 7·5 % of protein had 13 % of these linkages broken, whereas 29 % were broken when the DNA contained 50 % of protein.

DISCUSSION

The DNA–protein complexes isolated by the use of various concentrations of sodium benzoate have the surprising property of being soluble in water and in 0·1 M-sodium chloride solutions even when 30–50 % of protein is present. Moreover, these complexes are sufficiently stable to be extractable by 2-methoxyethanol from phosphate buffer in exactly the same manner as free DNA. Cysteine is present and although Crampton, Stein & Moore (1957) and Davison (1957) found this amino acid in calf-thymus histone, Brunish, Farley & Luck (1951) did not find it in rat-liver nucleohistone. The inability of *p*-aminosalicylate to remove all the protein from a DNA preparation containing 50 % of protein is unexpected, although it is possible that the protein has been modified during the extraction with phenol. The viscosity of the solutions was much higher when proteins were present and hence a high viscosity alone cannot be a criterion of the purity of DNA.

The ability of the diethyldithiocarbamate ion to release DNA with considerably less protein than the DNA released by the diethylacetate ion, and the activity of 2-nitroso-1-naphthol-4-sulphonate compared with 1-naphthol-4-sulphonate at 0·03 M-concentration could be related in both cases to the

complexing powers of diethyldithiocarbamate and 2-nitroso-1-naphthol-4-sulphonate with metals.

Fluoride and azide produce DNA with similar amounts of residual amino acids and both these ions form complexes with metals. In this respect it is relevant that Felix, Jilke & Zahn (1956) prepared highly polymerized DNA from bull testes by treatment at 70° with saturated sodium chloride and 5% sodium fluoride solution.

The DNA isolated after treatment with *cyclo*hexanediamine-*NNN'N'*-tetra-acetate has relatively high quantities of aspartic and glutamic acids. Although this compound is used in 0·15M-concentration it appears to be acting more like an aliphatic anion than a compound with chelating properties, and it is noteworthy that ethylenediamine-*NNN'N'*-tetra-acetate did not liberate DNA from liver in conjunction with phenol.

It seems reasonable to assume from these and the previous results that neither the ability of these salts to complex with metals nor the power to combine with proteins is alone sufficient to release DNA free from protein out of rat-liver cells, but that both capacities are essential. The amount of protein left then would be a measure of the ability of the anion both to complex with the metal(s) concerned and to interact with the nuclear proteins. By choosing a salt and a particular concentration, DNA with almost any amount of protein could be obtained.

That the combined abilities to complex with metals and to interact with proteins are essential is demonstrated by the liberation of DNA by the action (in conjunction with phenol) of naphthalene-2-sulphonate (0·15M) and ethylenediamine-*NNN'N'*-tetra-acetate (0·026M), neither of which separately released any quantity of DNA from rat liver. Azide (0·03M), however, made little difference when used in combination with benzoate (0·28M). There are two reasonable explanations of these results: either the chromosomes contain some DNA linked through a metal bond to the protein or the complexing agents prevent the DNA from acquiring metal ions and subsequently protein by a secondary reaction. It has been demonstrated previously that DNA could be removed from solution by collagen which had been treated with certain bivalent metal ions, but the reaction was slow and only 50% of the DNA was absorbed during 14 days. But when DNA which was free from protein was homogenized with rat liver in the presence of 0·14M-benzoate the total DNA isolated had only about 3% of amino acids, whereas DNA isolated directly in the presence of 0·14M-benzoate contained about 30% of amino acids. It seems unlikely then that DNA combines with any protein released through breaking down the cells under the conditions used.

Moreover, calf-thymus DNA does not interact with bovine serum albumin to any extent unless the DNA has been heated first (Zubay & Doty, 1957), and mixtures of DNA and protein which did interact unspecifically would be expected to be insoluble because the cross-linking would be random. This is the case when calf-thymus DNA is mixed with histones (Bernstein, 1956). The protein moiety of such complexes may be expected to have a relatively high arginine content since the phosphate groups of the liberated DNA would be the most obvious points of attachment. In fact the DNA–protein complexes isolated from rat liver are soluble in water and have lower arginine contents than histones present in the nucleus (Kirby, 1957, Table 5), and the analytical values are more reasonably consistent than might have been expected from arbitrary mixtures of DNA and proteins.

It would be unwise to place too much reliance on the analytical values of the amino acid in view of the known limitations of the method used. Davison & Butler (1954), using the same method, found variations up to ±10%, and similar variations have been encountered here. However, the analyses do show that lysine and arginine are not the predominant amino acids in these proteins and indeed the amounts of glutamic and aspartic acids are frequently of the same order as the basic amino acids. The analytical values bear some similarities to those described by Davison (1957) for certain fractions of histones which have been separated on columns of carboxymethylcellulose. It is not surprising that different salts produce DNA–proteins with varying amounts of amino acids in view of the known heterogeneity of nucleohistones (Stedman & Stedman, 1950; Butler, Davison, James & Shooter, 1954; Grégoire & Limozin, 1954; Davison, James, Shooter & Butler, 1954; Davison & Butler, 1954; Daly & Mirsky, 1955; Crampton, Moore & Stein, 1955; Luck *et al.* 1956; Crampton, Stein & Moore, 1957; Davison, 1957).

It must be emphasized that these results apply only to rat-liver DNA and there is no reason to assume that phenol and *p*-aminosalicylate will produce DNA free from protein from all tissues and organisms. In fact this particular combination does not produce DNA free from protein from certain rat tumours or from wheat germ (results to be reported later), and Kay (1956) has reported that phage 1, which is active on *Salmonella typhi*, is inactivated and loses its DNA by reaction with phosphate, citrate and ethylenediamine-*NNN'N'*-tetra-acetate. Calcium and magnesium ions protect the phage against inactivation. It is quite possible therefore that a number of agents or combination of agents may have to be tried before a suitable system is found for other organisms.

Since DNA isolated from mammalian sources has no demonstrable biological activity it is necessary to compare samples by physical methods. The $\epsilon_{(P)}$ value (Chargaff, 1955) is an indication that DNA prepared by the phenol method has the same optical properties as samples prepared by other methods. The increase of $\epsilon_{(P)}$ with increasing amounts of protein may be due to scattering of the light, and this is almost certainly the case with DNA containing 30–50% of protein, where the solutions were opalescent.

Dr M. H. F. Wilkins and his co-workers have made a crystallographic examination by X-rays of DNA prepared from rat liver by the actions of *p*-aminosalicylate, *cyclo*hexanediamine-*NNN'N'*-tetra-acetate and diethylacetate ions. All the samples showed the 'A' pattern characteristic of the undenatured sodium salt of DNA (Wilkins, Seeds, Stokes & Wilson, 1953), and the best photographic pattern from the specimen prepared by the action of *cyclo*hexanediamine-*NNN'N'*-tetracarboxylate was not distinguishable from the pattern produced by calf-thymus DNA made by standard methods. Clearly phenol does not disintegrate the regular structure of the isolated DNA molecules and their ability to arrange themselves in a crystalline structure. Also the same crystalline structure is produced when the samples contain protein.

The sedimentation coefficient–distribution curves are further evidence that DNA prepared from rat liver by the phenol method is similar to other samples (Shooter & Butler, 1956). The rather remarkable feature was the relatively small difference in the weight-average sedimentation coefficient of the samples with 8 and 50% of protein. Since a relatively small proportion of the protein is affected by chymotrypsin it appears likely that most of this protein must be along the length of the DNA molecules and is not involved in cross-linking. This hypothesis would account for the observed solubility of the preparations.

SUMMARY

1. Deoxyribonucleic acid has been prepared from rat liver by the action of a number of salts in conjunction with phenol. The amount of residual protein attached to the deoxyribonucleic acid depends upon the salt used.

2. *p*-Aminosalicylate and diethyldithiocarbamate have been the only salts to produce a deoxyribonucleic acid nearly free from protein.

3. The ability to complex with metals, in addition to the power to interact with proteins, is required to liberate deoxyribonucleic acid with very little protein from rat liver. Neither naphthalene-2-sulphonate nor ethylenediamine-*NNN'N'* tetra-acetate separately liberates any appreciable quantity of deoxyribonucleic acid but the two salts will do so when used together.

4. No protein was taken up on deoxyribonucleic acid which was mixed with a rat-liver homogenate in the presence of 0·14 M-benzoate.

5. The relevance of these experiments to the nature of the deoxyribonucleic acid–protein bonding is discussed.

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The Metabolism of Chlorotic Leaves

1. AMINO ACIDS

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Considerable attention has been paid to the enhanced amounts of free amino acids in plant leaves affected by iron-deficiency chlorosis. Bennett (1945) considered this to be the most typical symptom of iron-deficient leaves, and Iljin (1951) noted a marked increase of amino acids in the leaves of plants affected by 'lime-induced' chlorosis, a form of iron deficiency.

Using the technique of paper chromatography, Demetriades (1956) demonstrated the increase of amino acids which occurred in iron-deficient leaves of *Hibiscus esculentus*, an increase in arginine being especially prominent. Iron-deficient blueberry leaves were found to contain up to ten times more arginine (determined with Sakaguchi's reagent) than the healthy leaves (Holley & Cain, 1955). This was true also of leaves in which an iron deficiency was induced by toxic concentrations of nickel or cobalt (Cain & Holley, 1955).

Increases in arginine have similarly been recorded for the chlorophyll-deficient areas of variegated *Pelargonium* leaves by Euler & Burstrom (1933), but this was not true for the variegated leaves of *Euonymus* or *Tradescantia*. Bennett (1945) recorded increased free amino acids in variegated leaves and also in virus-infected leaves. Drever & Larv (1954) found a general increase of free amino acids in the leaves of peach affected by Western X virus; proline and pipecolinic acid were present only in the virus-affected leaves, a fact noted by others (Diener & Dekker, 1954). Similar increases in free amino acids have been recorded by Vavrich (1952) for virus-infected sugar-beet leaves and for leaves of *Acalypha indica* affected by mosaic virus (Laloraya, Govindjee & Raja Rao, 1955).

Young tissues have been found to contain more amino acids than mature tissues. Allsopp (1948) found an abundance of free amino acids in the growing points of ferns and much smaller amounts in the leaves. Steward, Wetmore, Thompson & Nitsch (1954) found that the free amino acids in *Lupinus* leaves decreased as the leaves expanded, especially lysine, arginine, valine, leucine, phenylalanine and tryptophan. Similar results were obtained by Mansford & Raper (1954) for *Equisetum*, by McKee, Nestel & Robertson (1955) for the developing pea, by Shibamoto, Shoji & Tagawa (1955) for bamboo shoots and by Petronici (1956) for *Vicia faba* leaves. This effect of age on the free amino acids in plant leaves is also mentioned by Iljin (1951) and by Bennett (1945), both investigators noting that differences in amino acid patterns of healthy and chlorotic leaves were most striking in the spring.

Since leaves suffering from iron deficiency, genetical chlorosis or certain virus infections, and also young leaves, contain more phosphorus relative to iron and more potassium relative to calcium (DeKock & Hall, 1955), it was of interest to determine whether these large amounts of free amino acids were always found in leaves with high phosphorus:iron and potassium:calcium ratios, irrespective of the cause.

METHODS

Extraction. Samples of about 25 g. of leaf tissue from various sources were macerated for 3 min. in a Waring Blendor with 100 ml. of 70% acetone and allowed to stand overnight. After filtration, the residue was extracted with hot 0·01 N-HCl, filtered and combined with the first extraction after removal of the acetone by distillation. The pH was then adjusted to 7 and the volume of extract reduced to about 150 ml. *in vacuo* at 40°. The extract was then passed through a Zeo-Karb 225 column (28 cm. × 1·5 cm., H^+ form) at less than 10 drops/min., the amino acids being subsequently eluted from the column with aq. 2 N-NH_3 soln. after a water wash. The eluate was freed from excess of NH_3 by evaporation under reduced pressure, and the residue dissolved in water and made up to a suitable volume (10–50 ml.): propan-2-ol was incorporated to give a final concentration of 10%.

Paper chromatography. The amino acids were separated by two-dimensional paper chromatography on sheets of Whatman no. 1 filter paper (28·5 cm. × 23·5 cm.). The amino acid solutions were applied to the paper by means of an automatically adjusting micropipette (Meinhard & Hall, 1950). Solvents used were phenol–ethanol–water (3:1:1, by vol., containing 0·05% of 8-hydroxyquinoline) in an atmosphere containing NH_3, and butan-1-ol–acetic acid–water (30:6:14, by vol.), both mixtures being single-phase systems at 18°. The ascending-boundary technique (Williams & Kirby, 1948) was employed for the phenol run along the smaller dimension of the paper. After the run,

which required about 16 hr., the papers were dried, first in a current of air at room temp. and then in an oven at 80°. For the second dimension it was necessary, in order to obtain a sufficiently long run, to use the descending technique (Consden, Gordon & Martin, 1944), and to carry the solvent from the trough to the chromatogram a strip of filter paper was tacked temporarily with cotton thread to the leading edge of the sheet; the trailing edge was serrated (Hird, 1949). A run of about 20 hr. duration at 18° was required. The papers were dried in a current of air at room temp. for 18–20 hr. and then dipped in a solution of ninhydrin (0·2%) in acetone and heated at 65° for 20 min. Two standard papers with known amounts of the amino acids to be determined, at levels of about 0·02 and 0·08 μmole, were run simultaneously and under the same conditions as the samples.

Determination of amino acids. Paper strips, 2·5 cm. wide, containing the amino acid spots were cut from the chromatograms after being lightly demarcated in pencil with the aid of a Perspex template. The strips, which might contain one or two spots, were scanned in a semi-automatic recording reflectance densitometer (model 2 SR, Joyce, Loebl and Co., Newcastle upon Tyne) and an absorption curve was obtained for each spot. The area under the curves, obtained by taking the product of the height and the width at half the height, was plotted against the weight of amino acid for the standard spots, giving a reference curve for each amino acid from which the amount in the samples could be obtained. Since the relationship between the area under the absorption curve and the weight of amino acid was not usually strictly linear, although nearly so, particularly for small amounts, it was necessary for maximum accuracy to adjust the samples so that the weight of each amino acid to be determined was well within the reference curve for the standards. It was usually necessary to prepare two chromatograms for each sample, one for those amino acids present in relatively high concentrations and another for the remainder.

Some amino acids were best determined by special methods. Tryptophan (when present) was determined on one-dimensional chromatograms after a short ascending run in the butanol–acetic acid solvent. The dried papers were dipped in a 1% solution of *p*-dimethylaminobenzaldehyde in acetone–10 N-HCl (9:1, v/v) and suspended for 80 min. at room temp. (18°). The violet tryptophan spots were then scanned in the densitometer as described above. Standards were run along with the samples. Histidine was determined on similar chromatograms, after spraying with a solution of diazotized sulphanilic acid [0·3 % in 8% (w/v) HCl] followed by 20 % Na_2CO_3 (Bray, Thorpe & White, 1950), by means of the reflectance densitometer. Comparative standards were used.

Proline was determined by the densitometer on one-dimensional chromatograms run in the butanol–acetic acid solvent, dipped in solution of isatin (0·2% in acetone), and heated at 75° for 4 min.

Replication experiments showed that the accuracy of determination of individual amino acids by this method was better than ±20%. Methionine, cystine, β-alanine and citrulline were sometimes detected but only in minor quantities and were not determined. Glycine was not well separated from serine and glutamine by the methods used and may sometimes have escaped detection, but it was never present in large quantities.

RESULTS

Comparison of extraction procedures. In order to test the accuracy of the extraction method, the leaves of mustard plants (*Sinapis alba*) grown under identical conditions in a greenhouse were divided into three portions, two of which were extracted as described above and the third was boiled in 200 ml. of water for 3 min., the leaves then being blended and filtered; after removal of acetone, subsequent procedures for the three samples were identical. Table 1 gives the results of the trial, showing that both the extraction procedure and reproducibility are satisfactory, although the γ-aminobutyric acid figure is somewhat higher in the water extract.

Iron-deficiency chlorosis. Bracken (*Pteris aquilina*) was gathered from calcareous and acid soils at Ullapool, Wester Ross, the former sample showing severe lime-induced chlorosis. The results are shown in Table 2, together with normal and iron-deficient spinach beet (*Beta vulgaris*) grown in sand culture in the greenhouse and healthy and nickel-toxic pea leaves (*Pisum sativum* var. Onward) grown in soil. Striking increases occurred in aspartic and glutamic acids, serine and most other amino acids in chlorotic bracken. In both the iron-deficient spinach beet and peas the increases in various amino acids were not so marked. In

Table 1. *Some free amino acids of mustard leaves*

Results are expressed as mg./100 g. of fresh leaf. Samples nos. 1 and 2 were extracted with acetone and no. 3 was extracted with boiling water. The upper and lower figures refer to chromatograms with slightly different loadings. Amino acids also present in mustard leaves but not determined were: arginine, asparagine, leucine, isoleucine, lysine, phenylalanine, tyrosine. Histidine and tryptophan were absent.

	Sample no.		
	1	2	3
Aspartic acid	29·1 34·6	27·2 22·2	34·5 27·7
Glutamic acid	58·4 63·6	64·1 58·2	58·2 57·4
Glutamine	7·2	7·7	10·9
Serine	27·2	30·9	28·4
Threonine	9·6 9·3	8·5 7·8	11·4 9·4
Alanine	13·9	10·1	11·0
γ-Aminobutyric acid	6·4 7·8	8·0 6·7	16·4 12·5
Valine	6·0	8·7	9·0
Proline	111·0*	114·0*	140·0*
Carboxyl nitrogen	37·6	33·4	36·0
Kjeldahl nitrogen	65·0	65·0	66·0

* Determined with isatin, after chromatography.

Table 2. *Free amino acids of healthy and iron-deficient leaves*

—, Not detected; P, present in fair quantity but not determined. Results are expressed as mg./100 g. of fresh leaf for both normal (N) and iron-deficient (D) leaves.

	Bracken (23)*		Spinach beet (50)*		Peas (43)*	
	(N)	(D)	(N)	(D)	(N)	(D)
Aspartic acid	0·7	10·8	6·0	13·2	9·0	7·0
Glutamic acid	1·0	80·4	17·3	26·5	14·1	26·9
Serine	1·3	59·0	3·9	15·3	2·5	4·5
Threonine	10·5	70·4	1·6	5·5	16·0	45·7
Alanine	5·7	118·1	4·9	6·6	3·0	4·5
Tyrosine	2·8	23·8	1·0	4·1	0·6	0·6
γ-Aminobutyric acid	22·9	54·0	2·1	8·6	1·9	3·7
Valine	38·0	208·3	2·3	3·1	1·3	3·9
Leucine–isoleucine	70·0	258·0	4·1	6·2	1·4	2·5
Phenylalanine	178·0	418·2	0·7	1·4	3·1	6·6
Histidine	6·5	P	2·8	3·9	—	—
Lysine	0·8	3·3	1·4	1·0	1·2	4·2
Arginine	2·1	5·6	3·8	3·3	18·7	24·9
Asparagine	0·4	66·5	6·5	19·6	4·4	12·5
Glutamine	7·8	7·4	0·7	2·3	2·2	0·4
Proline	6·0	40·3	44·1	48·1	4·4	11·1
Tryptophan	38·5	30·5	1·3	1·9	0·7	1·1
Total	393·0	1454·6	104·5	170·6	84·5	160·1

* Sample reference number (Table 8).

Table 3. *Free amino acid of mustard leaves* (78)* *grown in nutrient solutions A, B, C and D containing* 0·1, 0·5, 2·5 *and* 12·5 *p.p.m. of iron respectively*

—, Not detected; T, detected but too weak for determination.

	Concn. (mg./100 g. of fresh leaf)			
	A	B	C	D
Aspartic acid	43·6	31·1	36·2	32·7
Glutamic acid	136·3	79·8	54·1	51·1
Serine	24·7	24·1	12·3	15·3
Threonine	10·5	8·6	8·0	7·4
Alanine	20·9	8·9	6·5	8·3
Tyrosine	3·0	2·7	2·2	1·0
γ-Aminobutyric acid	9·1	4·7	3·6	4·5
Valine	12·1	6·0	6·7	5·4
Leucine–isoleucine	9·4	5·2	2·6	4·4
Phenylalanine	2·0	T	T	T
Lysine	2·5	0·7	T	—
Arginine	70·1	18·2	5·2	T
Asparagine	15·8	5·1	4·5	1·7
Glutamine	8·0	8·8	3·8	3·0
Histidine	5·0	—	—	—
Proline	39·5	37·7	31·8	28·2
Total	412·5	241·6	177·5	163·0

* Sample reference number (Table 8).

Table 3 the free amino acids of mustard (*Sinapis alba*) plants grown in nutrient solutions containing 0·1, 0·5, 2·5 and 12·5 p.p.m. of iron as the *NN'*-ethylenebis-(2-*o*-hydroxyphenyl)glycine chelate are given. The differences in free amino acids were most marked at the lower levels of iron; at the higher levels there were only small differences. The decrease in arginine was especially marked.

Genetical chlorosis. Normal green leaves and completely albino leaves were obtained from a variegated plant of *Bougainvillea glabra* in the greenhouse of the Macaulay Institute. Two sets of samples were collected on successive years. The results are presented in Table 4, and show the remarkable accumulation of arginine which occurs in the chlorotic leaves. Histidine was also much higher in the first albino sample, but actually lower in the second sample.

Effects of age. The decrease of free amino acids which takes place when cabbage leaves age is shown in Table 5. Analyses of the unexpanded 'heart' leaves and the expanded green leaves of four cabbage varieties, Greyhound, Winningstadt, January King and Myate are given. The difference between varieties is shown, as also is the greatly enhanced amount of arginine present in the young leaves.

Grass samples (mainly *Cynosurus cristatus*) taken in May 1956 and July 1956, and samples of beech leaves (*Fagus sylvatica*) taken in June 1956 and August 1956 showed abundant amino acids in the young leaves and marked decrease in the older leaves, to such an extent that only proline was detected in older grass by the method employed (Table 6).

Virus infection. Samples of healthy and diseased sugar beet (*Beta saccharifera*) affected by virus yellows, collected on 2 successive years, are shown in Table 7. The yellow leaves were also found to be boron-deficient. It is apparent that such virus-infected leaves contain much the same amounts of free amino acids as do the healthy leaves. A similar

picture was found when chlorotic leaves of mustard plants grown in high concentrations of ferric ethylenediaminetetra-acetate were compared with healthy green leaves.

Table 8 represents the inorganic analyses of the various samples quoted, together with the calculated phosphorus:iron and potassium:calcium ratios. The sodium and magnesium values have been included so that ratios of univalent to bivalent cation can be calculated if desired. Sample numbers are given in parentheses. No analyses were obtained for the grass samples (39, 42).

Table 4. *Free amino acids of leaves of variegated* Bougainvillea

T, Detected but too weak for determination.

	Concn. (mg./100 g. of fresh leaf)			
	(38)*		(52)*	
	Green	Albino	Green	Albino
Aspartic acid	26·3	29·3	14·2	22·1
Glutamic acid	45·5	28·4	35·5	21·6
Serine	10·6	5·0	4·4	3·2
Threonine	5·7	7·2	3·5	7·5
Alanine	6·0	4·1	6·3	4·0
Tyrosine	1·5	1·2	1·6	1·4
γ-Aminobutyric acid	7·4	5·9	3·8	4·8
Valine	1·7	3·4	2·2	4·6
Leucine–isoleucine	4·4	6·9	1·7	6·6
Phenylalanine	3·0	3·0	2·3	8·9
Histidine	12·6	73·5	10·4	4·2
Lysine	3·0	5·7	4·4	12·0
Arginine	37·1	157·0	24·5	213·0
Asparagine	7·6	2·1	4·8	6·0
Glutamine	3·2	0·7	1·8	2·6
Tryptophan	T	T	4·2	8·0
Proline	27·0	23·3	38·0	40·5
Total	202·6	356·7	163·6	371·0

* Sample reference number (Table 8).

DISCUSSION

Since methods of estimation of individual amino acids may be inaccurate to the extent of 20%, and in view of the results of replicated extractions given in Table 1, it is considered unlikely that differences between samples of less than 50% are significant. However, differences between the free amino acids of normal and chlorotic leaves are frequently very large and certainly greater than 100%, which leaves little doubt of their significance.

That deficiency of iron in leaves does not cause merely one or a few amino acids to increase, but rather an overall increase is shown in Table 2. It is immaterial whether the iron deficiency was caused by absence of iron (spinach), by its unavailability in calcareous soils (bracken) or by heavy-metal toxicity (pea). Moreover, it would appear impossible to single out one particular amino acid which shows consistent trends in the three plants studied. Thus although Holley & Cain (1955) found large increases of arginine in iron-deficient and 'cobalt-toxic' blueberry leaves, this is not shown by any of the above plants, nor is it shown by iron-deficient tomato plants (Possingham, 1956).

Table 5. *Free amino acids of young (W) and mature (G) leaves of four varieties of cabbage*

—, Not detected; T, detected but too weak for determination.

	Concn. (mg./100 g. of fresh leaf)							
	Greyhound (45)*		Winningstadt (53)*		January King (61)*		Myate (71)*	
	(W)	(G)	(W)	(G)	(W)	(G)	(W)	(G)
Aspartic acid	—	T	61·5	18·1	53·0	33·1	41·2	18·0
Glutamic acid	—	T	72·1	30·8	131·4	51·8	135·4	23·1
Serine	—	—	22·0	5·7	23·5	7·6	30·6	6·9
Threonine	—	—	7·1	4·3	18·5	7·5	12·8	4·9
Alanine	—	—	24·4	6·4	35·6	25·6	32·6	8·0
Tyrosine	—	—	3·4	0·6	1·0	—	3·4	—
γ-Aminobutyric acid	0·1	—	13·0	6·3	58·3	30·7	4·3	4·4
Valine	—	—	24·4	8·4	11·2	7·0	11·9	2·5
Leucine–isoleucine	—	—	18·7	3·7	6·9	5·0	11·7	2·1
Phenylalanine	—	—	7·9	2·4	5·1	1·8	2·6	T
Histidine	—	—	15·1	1·3	—	—	13·1	—
Lysine	1·8	0·1	5·2	0·4	—	—	7·2	1·2
Arginine	13·2	1·5	119·0	5·9	264·0	53·0	32·2	2·2
Asparagine	—	—	28·8	4·0	58·3	9·9	40·8	T
Glutamine	—	–	18·5	1·0	53·1	—	15·2	—
Pipecolinic acid	—	—	1·0	4·5	T	T	T	T
Tryptophan	T	—	3·6	1·1	—	—	1·6	T
Proline	2·2	2·2	80·7	24·6	315·0	231·0	11·0	6·7
Total	17·3	3·8	526·4	129·5	1034·9	464·0	407·6	80·0

* Sample reference number (Table 8).

Mustard plants (Table 3) do, however, show a progressive decrease in arginine with improved iron status. This has been recorded by Steinberg (1955) for tobacco plants.

Table 6. *Free amino acids of old and young grass and beech leaves*

—, Not detected; T, detected but too weak for determination.

	Concn. (mg./100 g. of fresh leaf)			
	Grass (39)* (young)	Grass (42)* (old)	Beech leaves (40)* (young)	Beech leaves (44)* (old)
Aspartic acid	9·8	—	32·0	5·9
Glutamic acid	33·6	—	21·3	23·1
Serine	5·6	—	3·2	1·2
Threonine	1·5	—	0·4	0·5
Alanine	14·6	—	4·7	2·5
Tyrosine	1·1	—	5·7	0·7
γ-Aminobutyric acid	5·3	—	7·4	2·7
Valine	4·3	—	1·3	1·0
Leucine–isoleucine	2·6	—	1·6	0·6
Phenylalanine	1·7	—	1·4	1·7
Histidine	—	—	1·9	—
Lysine	0·3	—	0·8	T
Arginine	0·6	—	0·4	—
Asparagine	1·3	—	23·6	1·4
Glutamine	—	—	0·8	0·4
Tryptophan	—	—	0·8	—
Proline	24·2	3·6	6·6	6·6
Total	106·5	3·6	113·9	48·3

* Sample reference number (Table 8).

The increase in arginine in the albino leaves of variegated *Bougainvillea* is, however, quite striking (Table 4). Here a general increase of all amino acids is not apparent; in fact amino acids such as glutamic acid and alanine were higher in the green leaves. Arginine does not, however, show an increase in all variegated leaves (Euler & Burstrom, 1933).

The immature leaves of the various varieties of cabbage (Table 5) also showed large accumulations of arginine. It is, however, apparent that immature or young tissues contain larger quantities of most amino acids, as can be seen in young and old grass and also in beech leaves (Table 6). The process of ageing of leaves thus appears to be accompanied by a progressive disappearance of free amino acids. This is in apparent contradiction to the results of Yemm (1956) who reported an increase of amino acids in senescent leaves. It must be remembered, however, that final death of a tissue such as a leaf is preceded by a 'climacteric bump' in which both respiration rate and free amino acids increase.

The results obtained with chlorotic leaves of virus-infected sugar beet together with chlorotic leaves of iron-toxic mustard (Table 7) are in apparent disagreement with the results discussed above and with those of other workers. Reference to Table 8, however, shows that whereas the previous results are for leaves of which the chlorotic ones show higher ratios of phosphorus to iron and

Table 7. *Free amino acids of healthy and virus-infected leaves of sugar beet and healthy and 'iron-toxic' mustard leaves*

Gr, Green; Y, yellow; —, not detected; T, detected but too weak for determination.

	Concn. (mg./100 g. of fresh leaf)					
	Sugar beet (27)*		Sugar beet (48)*		Mustard (28)*	
	(Gr)	(Y)	(Gr)	(Y)	(Gr)	(Y)
Aspartic acid	0·7	T	3·9	5·8	0·8	1·2
Glutamic acid	2·8	T	10·5	19·3	6·0	6·6
Serine	0·9	—	2·8	4·6	1·6	3·3
Threonine	0·9	T	0·9	2·6	2·0	2·7
Alanine	0·8	T	3·4	1·9	2·4	4·2
Tyrosine	0·9	0·5	11·7	10·1	0·6	0·3
γ-Aminobutyric acid	0·9	0·2	6·7	7·4	1·0	1·2
Valine	2·4	—	12·2	15·6	2·7	3·0
Leucine–isoleucine	3·3	—	15·8	13·8	2·8	2·2
Phenylalanine	3·4	0·5	17·7	7·1	0·7	0·6
Histidine	2·2	2·0	2·8	3·2	0·3	0·1
Lysine	0·6	0·1	3·7	1·4	0·3	0·3
Arginine	0·6	0·3	6·1	3·1	1·6	1·1
Asparagine	1·4	T	3·2	4·0	0·1	0·2
Glutamine	2·7	T	2·7	6·0	—	—
Pipecolinic acid	—	—	—	—	0·2	0·1
Tryptophan	2·6	1·9	9·9	10·7	0·6	T
Proline	1·0	—	6·8	7·3	7·2	7·4
Total	28·1	5·5	120·8	123·9	30·9	34·5

* Sample reference number (Table 8).

Table 8. *Analyses (%) of the ash of various plant leaves*

Y, Young; O, old; W, white; G, green; Chl, chlorotic. Sample numbers are given in parentheses.

		P	Fe	K	Ca	Na	Mg	P/Fe	K/Ca	Total amino acid	Ash (%)
Beech (40)	Y	5·72	0·153	21·1	7·3	0·7	3·6	37·4	2·89	113·9	4·4
Beech (44)	O	3·48	0·180	19·8	11·8	1·2	4·0	19·3	1·68	48·3	5·0
Bougainvillea (38)	W	2·68	0·283	33·1	8·6	0·2	1·4	9·5	3·85	356·7	20·0
	G	0·72	0·146	13·6	26·7	0·4	1·8	4·9	0·51	202·6	24·0
Bougainvillea (52)	W	2·81	0·111	29·2	7·7	0·2	2·2	25·3	3·79	371·0	19·2
	G	2·5	0·125	14·2	25·2	0·3	2·4	20·0	0·56	163·6	20·6
Bracken (23)	Chl	4·28	0·087	12·1	20·0	2·4	2·1	49·2	0·61	386·5	5·3
	G	3·76	0·048	13·9	21·6	4·8	2·0	78·3	0·64	1454·6	9·3
Cabbage (45) (Greyhound)	W	6·49	0·070	34·7	4·6	0·9	2·0	92·7	7·54	17·3	9·7
	G	2·82	0·076	16·1	18·6	0·7	1·8	37·1	0·87	3·8	18·5
Cabbage (53) (Winningstadt)	W	6·57	0·067	37·3	2·1	0·6	2·4	98·1	17·76	526·4	8·2
	G	3·65	0·058	18·7	16·7	0·8	1·7	62·9	1·12	129·5	12·0
Cabbage (61) (January King)	W	6·67	0·065	39·0	4·0	0·19	2·3	102·6	9·75	1034·9	8·0
	G	2·67	0·033	24·4	16·5	0·69	1·1	80·9	1·48	464·0	13·1
Cabbage (71) (Myate)	W	8·13	0·099	40·9	1·9	0·38	2·2	82·1	21·5	407·6	8·1
	G	1·78	0·033	14·9	15·9	2·2	1·5	53·9	0·9	80·0	12·0
Mustard (28)	G	3·11	0·050	10·6	24·1	0·2	4·6	62·2	0·44	30·9	—
	Chl	0·81	0·080	2·0	32·1	0·5	3·7	10·1	0·06	34·5	—
Spinach beet (50)	Chl	7·96	0·207	19·1	6·4	3·6	3·6	38·5	2·97	104·5	12·1
	G	3·20	0·058	29·6	2·9	5·7	3·5	55·2	10·21	170·6	12·9
Sugar beet (27)	G	1·11	0·046	20·5	5·1	17·1	5·6	24·1	4·02	28·1	10·1
	Chl	0·97	0·089	15·4	7·6	13·3	2·3	10·9	2·03	5·5	18·1
Sugar beet (48)	G	2·43	0·083	24·0	4·5	13·2	2·3	29·3	5·33	120·8	11·4
	Chl	1·73	0·110	23·3	6·0	12·2	2·4	15·7	3·88	123·9	12·5
Pea (43)	Chl	1·9	0·076	17·2	13·7	2·4	3·7	25·0	1·26	84·5	10·5
	G	2·3	0·023	24·0	13·5	2·7	3·9	100·0	1·77	160·1	11·1
Mustard (78)	A	7·98	0·081	20·1	11·7	2·4	3·2	98·5	1·72	—	11·3
	B	4·29	0·088	20·2	14·2	2·8	3·1	48·8	1·42	—	11·4
	C	3·66	0·098	20·2	15·8	2·5	3·4	37·3	1·27	—	12·2
	D	3·64	0·117	19·5	16·9	2·6	3·2	31·1	1·15	—	12·3
Kale (54)	G	4·52	0·074	26·6	6·8	2·6	1·7	61·08	3·91	—	8·2
	W	6·29	0·086	35·8	2·8	0·9	1·7	73·14	12·79	—	8·9

potassium to calcium, in the chlorotic sugar-beet leaves affected by virus yellows and in iron-toxic mustard the reverse is true, the chlorosis being of an iron-toxic type, and in such chlorotic leaves it would appear that free amino acids are little altered.

The above studies seem to indicate a close relationship between the mineral analysis of the leaf and its content of free amino acids, the latter being high when the phosphorus:iron ratio is high and declining as the phosphorus:iron ratio declines, whether this is brought about by improving the iron status or as an ageing effect. Such a relationship between the content of free amino acids and 'active' iron (i.e. iron extractable from dried leaves by N-HCl) in leaves was pointed out by Bennett (1945) and by Holley & Cain (1955). Though our understanding of the metabolism of amino acids within the plant cell is far from complete, their intimate relationship with the organic acids is known (Steward & Pollard, 1957). It is the purpose of the next paper to compare the changes in the organic acids with the changes in amino acids.

SUMMARY

1. The free amino acid content of healthy and chlorotic leaves has been studied. Iron-deficient chlorotic leaves contained enhanced amounts of free amino acids, even when the iron deficiency was induced by heavy metals or was due to genetical causes.

2. In iron-toxicity chlorosis, however, the content of free amino acids was not very different from that of healthy leaves.

3. It would appear that the free amino acid content of leaves depends upon their iron status, as reflected by the phosphorus:iron ratio.

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The Metabolism of Chlorotic Leaves

2. ORGANIC ACIDS

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DeKock (1956) has pointed out that chlorosis in plant leaves, whether induced by excess of lime, by iron deficiency, by toxicity of heavy metals or by genetical or virological factors, usually appears to be characterized by higher ratios of phosphorus to iron and of potassium to calcium than are found in the normal green leaf. The levels of some of the organic constituents in normal and chlorotic leaves have been examined to determine whether any consistent distortion of the normal pattern occurs in chlorotic leaves, irrespective of the cause of chlorosis.

In a study of the free amino acids of such chlorotic and healthy leaves of various plants, it was found that significantly larger amounts of free amino acids were associated with tissues in which higher phosphorus:iron and potassium:calcium ratios were found (DeKock & Morrison, 1958).

Various plant leaves with high potassium contents have been shown to contain large amounts of citric acid (Cooil, 1948; Iljin, 1951; Kurchatov, 1940), whereas leaves with high calcium contents contain either large amounts of oxalic acid (Olsen, 1939; Chandler, 1937; Scharrer & Jung, 1953, 1954; Pierce & Appleman, 1943) or of malic acid (Cooil, 1948). McGeorge (1949) found a marked correlation between iron extractable with N-HCl ('active iron'), citric acid and oxalic acid in citrus and deciduous fruit-tree leaves, chlorotic leaves containing less active iron, more citric acid and less oxalic acid than green leaves. Determinations of the organic acids of healthy and chlorotic leaves used in the previous study (DeKock & Morrison, 1958) were therefore made to see if the citric acid:(malic plus oxalic acid) ratios would vary as the phosphorus:iron and potassium:calcium ratios.

METHODS

Samples were those used in the investigation of the free amino acids of chlorotic leaves (DeKock & Morrison, 1958). The effluent from the Zeo-Karb 225 column was run through an Amberlite-IR4B column, which absorbed the organic acids. The effluent was discarded. The organic acids were then eluted from the column with 200 ml. of aq. N-NH_3 soln., and the eluate was concentrated to a small volume by evaporation on a steam bath and made up to 10 ml. A portion of this was then used for analysis by partition chromatography.

Partition chromatography of plant organic acids. The non-volatile organic acids were separated by partition chro-

matography on a column of silica gel, a gradient-elution technique being used, and determined by titration with standard alkali.

Silica gel. This was prepared from sodium metasilicate essentially as described by Neish (1949), but in order to obtain sufficiently high flow rates on the column it was necessary before drying the gel to remove the finer particles by suspending the material repeatedly in water and decanting the supernatant liquid after the coarser material had settled.

Solvents. Solvents used were mixtures of $CHCl_3$ and technical grade *tert.*-amyl alcohol (2-methylbutan-2-ol) saturated with 0·1 N-H_2SO_4. Before use, the $CHCl_3$ was washed with water to remove ethanol, and the *tert.*-amyl alcohol was shaken with solid NaOH (5 g./l.) and redistilled to remove traces of acidic material. The mixtures generally employed were 2% and 50% (v/v) $CHCl_3$ in *tert.*-amyl alcohol (afterwards referred to as CA2 and CA50 respectively); these were equilibrated with 0·1 N-H_2SO_4 and passed through filter paper to remove suspended water droplets.

Preparation of column. The silica-gel column was contained in a glass tube 25 cm. long and 1·2 cm. internal diameter, constricted to 0·4 cm. at the lower end and having a B19 standard ground-glass joint at the upper end. Dry silica gel (4 g.) was mixed with 3·5 ml. of 0·1 N-H_2SO_4 and slurried with 20 ml. of $CHCl_3$. A small plug of cotton wool was packed into the bottom of the tube and the slurry was poured in. Excess of $CHCl_3$ was allowed to drain and any gel adhering to the tube above the liquid level was washed down with a little $CHCl_3$. The gel was then packed down by inserting a circle of filter paper with a diameter slightly greater than that of the tube and pressing down with a stainless-steel plunger which was a sliding fit for the tube.

Application of sample. A suitable volume (usually 0·5–2·0 ml.) of the sample solution was made just alkaline to thymol blue and evaporated to dryness on a steam bath. The residue was cooled and dissolved in 0·1 N-H_2SO_4 (0·5 ml.) and mixed with 0·7 g. of dry silica gel to give a free-flowing powder, which was transferred to the top of the column. It was washed down and slurried with a small volume (5 ml.) of $CHCl_3$ and packed under a filter-paper disk. The tube was then filled with solvent mixture CA2.

Gradient elution. The simple arrangement used for gradient elution, although evolved independently, is similar to that described by Kellie & Wade (1957), when a solvent gradient is applied to the column from two reservoirs, a recipient and a donor of suitable cross-sectional areas, connected by a siphon, the column being fed from the recipient reservoir which is stirred magnetically. Such a system delivers solvent of composition

$$C = C_2 - (C_2 - C_1)\,(1 - V)^{A_2\rho_1/A_1\rho_2},$$

where A_1 and A_2 are the respective areas of cross-section of recipient and donor reservoirs, C_1 and C_2 are the initial composition of the solvents, ρ_1 and ρ_2 are the densities of the solvents and V is the fraction of the total volume delivered (cf. Bock & Ling, 1954).

In practice the reservoirs were two glass bottles, of capacities 500 and 250 ml. such that the ratio A_2/A_1 was 0·637. The two solvents CA2 (C_1) and CA50 (C_2) had densities at 18° of 1·460 (ρ_1) and 1·145 (ρ_2) respectively, and the total volume of solvent was 500 ml., comprising 278 ml. of CA2 and 222 ml. of CA50. The bottles were connected by a siphon tube, and another siphon conducted the mixed solvent to the top of the column. By mounting the reservoirs about 80 cm. above the column sufficient pressure was obtained to give a suitable rate of flow (approx. 25 ml./hr.). The rate was controlled when necessary by a valve consisting of a stainless-steel rod sliding in a closely fitting glass capillary (Partridge & Brimley, 1951) fitted to the lower end of the tube. The effluent was collected in 4·5 ml. fractions by means of a siphon measuring device and an automatic fraction collector.

Titration of fractions. Fractions were titrated with 0·02 N-NaOH with thymol blue as indicator, by means of a microburette, the tubes being shaken vigorously during the titration.

Results obtained for the separation of a synthetic mixture of acid are shown in Fig. 1*A*, where the titre for each fraction is plotted against the fraction number. It can be seen that the technique gave a good separation with well-defined peaks of fumaric, succinic, malic and citric acid. The pairs, malonic acid–*trans*-aconitic acid and oxalic acid–*cis*-aconitic acid, coincided under these conditions, although it was later found that they could be separated from each other and from the acids previously mentioned by the use of 0·1 N-H_2SO_4 in 50% glycerol–water as the stationary phase (Fig. 1*B*). All results given were obtained with aq. 0·1 N-H_2SO_4 as the stationary phase. Recovery of individual acids was always better than 95%.

Oxalic acid. Because of its volatility, oxalic acid was determined by Baker's method as outlined by Palmer (1955) on separate samples. Although determinations by the silica-gel column frequently agreed very well with the chemical method, it could not always be relied upon and separate determinations were usually made.

Identification of acids. After elution from the silica-gel column, fractions were identified by paper chromatography (Buch, Montgomery & Porter, 1952). It was not always possible to determine the identity of acid in the peak with maximum about fraction 39, although both malonic and *trans*-aconitic acid could be present and have in a few instances been identified, as is indicated in the tables of results.

Total acidity. In a number of samples, the total organic acidity was determined by Palmer's (1955) method. It was found that Deacidite FF (50–100 mesh) could be substituted for Dowex 1, with equally good results, the procedure remaining unaltered. Values obtained by this method agreed very well with those obtained by the gradient-elution technique.

RESULTS

Comparison of extraction procedures. The reproducibility of the extraction was tested on mustard leaves grown in a standard nutrient medium with 2 p.p.m. of iron as ferric ethylenediaminetetraacetate. The procedure is described in the previous paper (DeKock & Morrison, 1958), the eluted acid from the IR 4b columns being separated by partition chromatography on silica gel. Results are given in Table 1.

As reproducibility was deemed satisfactory, the acetone-extraction procedure was adhered to.

Iron-deficiency chlorosis. Mustard plants grown in nutrient culture with 0·1, 0·5, 2·5 and 12·5 p.p.m. of Fe as the *NN'*-ethylenebis-(2-*o*-hydroxyphenyl)-glycine chelate were harvested at the flower-bud stage. The mature leaves were separated and organic acids estimated as before. Results are shown in Table 2.

Genetical chlorosis. Colourless (albino) and green leaves were harvested from a plant of *Bougainvillea glabra variegata*. The analysis of the organic acids are given in Table 3. The leaves of a variegated variety of kale grown in a garden soil were harvested

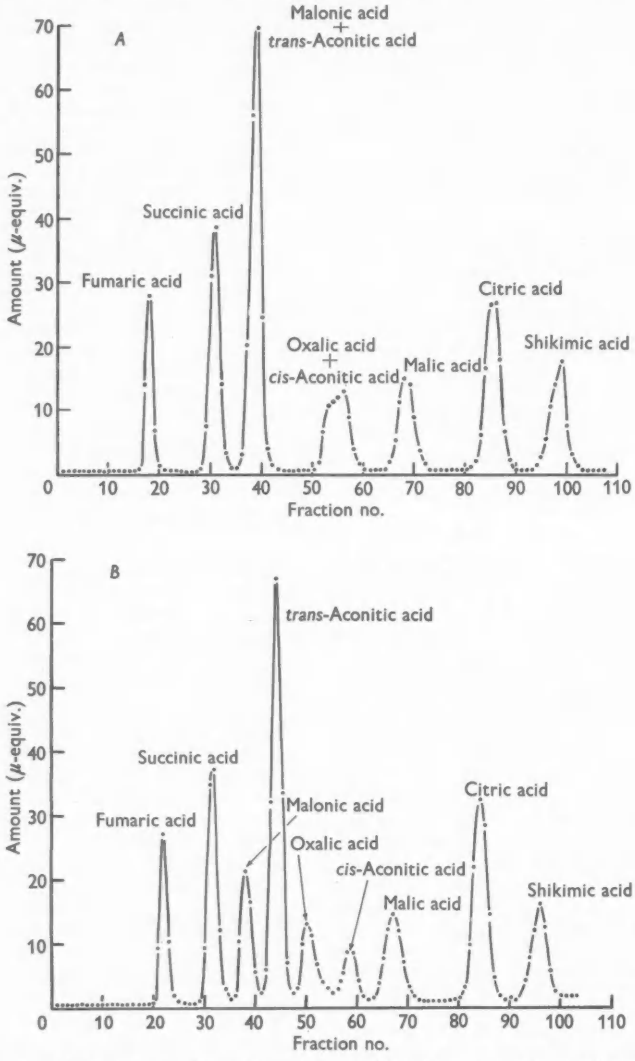

Fig. 1. Separation of synthetic mixtures of organic acids by partition chromatography on silica gel. Acid (μ-equiv.) in each fraction (4·5 ml.) is plotted against the fraction number. *A*, Separation obtained with 0·1 N-H_2SO_4 as stationary phase; *B*, separation with 0·1 N-H_2SO_4 in glycerol–water (1:1, v/v) as stationary phase.

and the green areas cut away from the chlorophyll-free areas.

Metal toxicity. Peas grown in a soil to which a considerable quantity of nickel had been added were severely chlorotic. The organic acid pattern of these leaves compared with that of healthy pea leaves is given in Table 4.

Ageing. Organic acids of the inner white leaves and the outer green leaves of four varieties of cabbage are shown in Table 5, and the analyses of young and old leaves of bracken are shown in Table 6.

Virological chlorosis. Leaves of sugar beet infected with virus yellows, and healthy leaves, were obtained from Cambridgeshire. The organic acid analysis of these leaves is shown in Table 7.

Mineral constituents. The analyses of the ash of the various leaves utilized in these organic acid studies are given in Table 8 of the preceding paper (DeKock & Morrison, 1958).

Table 1. *Organic acids of mustard* (70)* *leaves*

Samples A and B were extracted with acidified 70% acetone and sample C with boiling water. Oxalic acid was determined chemically, the remainder by silica-gel chromatography. For further details see text.

Acid	Content (m-equiv./kg. of leaf) A	B	C
Fumaric	0·6	0·5	0·7
Succinic	1·8	1·7	2·1
Unknown†	12·9	10·5	9·7
Oxalic	9·0	9·0	—
Malic	112·7	118·3	118·7
Citric	33·9	28·9	33·4
Citric: (malic + oxalic) ratio	0·30	0·24	0·28

* Sample reference number.
† Mainly malonic acid found.

Table 2. *Organic acid content of mustard* (78)* *leaves grown in nutrient solutions (respectively A, B, C and D) containing* 0·1, 0·5, 2·5 *and* 12·5 *p.p.m of iron*

Acid	Content (m-equiv./kg. of leaf) A	B	C	D
Fumaric	1·7	2·0	1·7	0·9
Succinic	2·6	1·8	1·9	2·1
Unknown†	11·6	11·7	11·5	11·0
Malic	38·9	58·4	68·1	78·5
Citric	19·5	23·5	23·5	26·8
Total acidity	74·3	97·4	106·7	119·3
Citric: malic ratio	0·50	0·40	0·35	0·34

* Sample reference number.
† Mainly malonic; identification by infrared spectrum.

Table 3. *Organic acids of albino* (*W*) *and green* (*G*) *leaves in genetical chlorosis*

Acid	Content (m-equiv./kg. of leaf) *Bougainvillea* (52)* (G)	(W)	Kale (54)* (G)	(W)
Fumaric	0·6	0·1	3·75	2·65
Succinic	1·0	0·4	5·5	3·05
Unknown†	1·9	0·2	1·85	1·65
Oxalic	93·8	25·0	0·9	0·7
Malic	1·9	1·0	57·65	24·4
Citric	1·5	4·1	14·9	34·1
Total acidity	100·7	29·8	84·55	66·55
Citric: (malic + oxalic) ratio	0·02	0·16	0·25	1·36

* Sample reference number (Table 8, DeKock & Morrison, 1958).
† Probably contains *trans*-aconitic acid or malonic acid or both.

Table 4. *Organic acids of leaves from normal* (*G*) *and 'nickel-toxic'* (*Chl*) *peas* (43)*

Acid	Content (m-equiv./kg. of leaf) (G)	(Chl)
Fumaric	8·8	6·5
Succinic	13·5	17·0
Unknown†	1·1	1·0
Oxalic	0·3	0·7
Malic	59·0	42·7
Citric	30·7	64·5
Total acidity	113·4	132·4
Citric: (malic + oxalic) ratio	0·51	1·49

* Sample reference number (Table 8, DeKock & Morrison, 1958).
† Probably contains *trans*-aconitic acid or malonic acid or both.

DISCUSSION

Increasing the iron supply to mustard plants in nutrient culture causes the phosphorus in the leaf ash to decrease, whereas the iron and calcium contents increase (Table 8, DeKock & Morrison, 1958). The total acidity of the leaf also increases owing to a considerable increase in malic acid and to a slight increase in citric acid. It will thus be seen that the citric acid: malic acid ratio is highest in the severely iron-deficient leaves and progressively declines as the iron status improves (Table 2). Both the phosphorus: iron and the potassium: calcium ratios show similar trends (Table 8, DeKock & Morrison, 1958).

The albino areas of both *Bougainvillea* and kale tissues, which are chlorophyll-free for genetical reasons, are found to contain more phosphorus and potassium and very much less calcium than the

Table 5. *Organic acids of the outer green (G) and inner white (W) leaves of four varieties of cabbage*

	Content (m-equiv./kg. of leaf)							
	Greyhound (45)*		Winningstadt (53)*		January King (61)*		Myate (71)*	
Acid	(G)	(W)	(G)	(W)	(G)	(W)	(G)	(W)
Fumaric	0·7	0·5	5·0	—	4·0	3·9	1·1	0·5
Succinic	1·5	0·4	22·7	1·0	1·0	2·0	0·9	1·1
Unknown†	0·6	1·35	4·5	5·9	1·3	8·0	0·8	7·6
Oxalic	0·2	0·4	2·8	0·9	—	0·4	—	—
Unknown	—	—	Trace	Trace	—	1·6	Trace	0·6
Malic	55·7	17·2	100·7	22·6	104·2	33·8	124·8	20·8
Citric	15·5	17·8	21·3	24·1	12·1	28·5	24·2	20·4
Total acidity	74·2	37·65	157·0	54·5	122·6	78·2	151·8	51·0
Citric: (malic + oxalic) ratio	0·28	1·01	0·21	1·03	0·12	0·83	0·19	0·98

* Sample reference number (Table 8, DeKock & Morrison, 1958).
† Probably contains *trans*-aconitic or malonic acid or both.

Table 6. *Organic acids of young (Y) and mature (O) bracken fronds*

	Content (m-equiv./kg. of leaf)	
Acid	Y (72)*	O (77)*
Fumaric	0·4	0·8
Succinic	1·5	0·8
Unknown†	2·4	1·8
Oxalic	0·2	0·2
Malic	4·8	7·0
Citric	4·5	4·4
Shikimic	6·3	4·2
Total acidity	20·1	19·2
Citric: (malic + oxalic) ratio	0·90	0·61

* Sample reference number (Table 8, DeKock & Morrison, 1958).
† Probably contains *trans*-aconitic and malonic acid.

Table 7. *Organic acids of healthy (G) and chlorotic (Chl) leaves of sugar beet* (48)* *infected with virus yellows*

	Content (m-equiv./kg. of leaf)	
Acid	G	Chl
Fumaric	2·2	1·4
Succinic	3·7	2·6
Unknown†	4·7	3·7
Oxalic	150·0	270·3
Malic	3·4	3·4
Citric	15·0	19·8
Total acidity	179·0	301·2
Citric: (malic + oxalic) ratio	0·10	0·07

* Sample reference number (Table 8, DeKock & Morrison, 1958).
† Malonic acid and *trans*-aconitic acid were identified in these fractions by paper chromatography.

corresponding green areas (Table 8, DeKock & Morrison, 1958). In both (Table 3), the citric acid content of the albino-leaf tissue is greater than that of the green-leaf tissue, whereas the reverse is true for the malic acid content. The oxalic acid content of both green and white tissue of kale leaves is quite low, but in *Bougainvillea* it is considerable and nearly four times as high in the green as in the albino. The total acids are also higher in the green tissues of *Bougainvillea*, but this difference is not so apparent in the variegated kale.

The iron-deficiency induced in pea plants (Table 8, DeKock & Morrison, 1958) by excess of nickel is accompanied by higher amounts of citric acid in the chlorotic leaves, whereas the malic acid is lower in these leaves compared with normal green leaves (Table 4).

The increase of malic acid which occurs when leaves age is seen in Table 5. The immature leaves of the cabbage 'heart' contain less total acid and much less malic acid than the green leaves. Citric acid is seen to be greater or only slightly less in the immature leaves than in the green leaves, so that the citric acid:malic acid ratio is once again high in leaves having high phosphorus:iron and potassium:calcium ratios. A similar situation can be seen in young and old bracken fronds (Table 6). In this instance, however, it is evident that shikimic acid makes a major contribution to the total acidity, and appears to be present in greatest amount in the younger leaves, thus behaving much like citric acid.

Sugar-beet leaves infected with virus yellows contain more oxalic acid than do the healthy green leaves (Table 7). Citric acid is slightly increased and the remaining acids are somewhat reduced. This result appears to contradict the previous findings for chlorotic leaves, but in this instance it will be seen that the chlorotic leaves contain more calcium and less potassium and phosphorus than the green leaves (Table 8, DeKock & Morrison, 1958). Thus the citric acid:(malic + oxalic acid) ratio is lower in the chlorotic leaves, as are also the phosphorus:iron and potassium:calcium ratios. Similarly, mustard leaves (sample 28, Table 8, DeKock & Morrison, 1958), which were chlorotic

owing to iron toxicity, were found to contain four times the malic acid content of the healthy green leaves, whereas the citric acid in these leaves was too small to be accurately determined.

Whereas Pierce & Appleman (1943), who estimated oxalic, malic and citric acid by chemical means, frequently found that 70–80% of the total acidity of the leaf could not be accounted for by these acids, no such discrepancies were found in the present studies, apart from skikimic acid in bracken and broad bean. Oxalic, malic or citric acid appears to be the major constituent. Fractions for fumaric, succinic, malonic or *trans*-aconitic acid were never present to any great extent. The *trans*-aconitic acid in these extracts probably arose from the conversion of *cis*-aconitic acid (Krebs & Eggleston, 1944).

From the results presented it would appear that a relation exists in leaves between the phosphorus: iron ratio, the potassium:calcium ratio and the citric acid:(malic + oxalic acid) ratio. The total organic acidity also appears to vary inversely with the total phosphorus in the leaf, as has been noted by others (Cooil, 1948; Vavrich, 1954; Ward & Petrie, 1940). Although the amount of carbonate has not been estimated in these leaves, its contribution to the cation–anion balance of the leaf is not very great (Scharrer & Jung, 1957).

In the previous part of this study it was shown that the free amino acids of leaf tissue were higher when the phosphorus content of the leaf was high (DeKock & Morrison, 1958). They therefore vary inversely as the total organic acids.

SUMMARY

1. Organic acids in healthy leaves and leaves chlorotic due to iron deficiency, heavy-metal toxicity, ageing, virus-infection and genetical constitution were quantitatively studied.

2. All types of chlorotic leaves contained more citric acid relative to malic or oxalic acid, except in virus yellows in sugar beet where the reverse is true.

3. The citric acid:(malic + oxalic acid) ratio of leaves appears to vary as the phosphorus:iron and potassium:calcium ratios. The total amount of organic acid also varied inversely as the phosphorus content of the leaf.

4. Green leaves apparently contain large quantities of either malic or oxalic acid.

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The Uptake and Metabolism of Amino Acids by Slices of Carrot

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It has been shown that several amino acids disappear from a solution which is in contact with slices of carrot tissue (Birt & Hird, 1956). Further, Webster (1954), Rheinhold & Powell (1956) and Kursanov (1956) have studied the uptake of certain amino acids by various plant tissues. However, actual evidence for the transport of amino acids into plant tissues against a concentration gradient is limited to that provided for the accumulation of histidine by carrot slices (Birt & Hird, 1956).

In an investigation of the mechanism responsible for the uptake of amino acids by carrot slices, it is essential to assess the contribution made by the metabolic destruction of these compounds to the

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apparent uptake as measured by disappearance from the external medium. Accordingly, the aim of the present investigation was to study the metabolic fate of a number of amino acids after they had been taken up by slices of carrot root. The term 'uptake' has been used to describe the disappearance from the external solution of the compound under investigation, that is adsorption on and entry into the tissue; the term 'accumulation' refers to the storage in the tissue of the chemically unaltered substance.

First, the proportions of the amino acids taken up that could be recovered from the tissue at the end of the experiment, and secondly, the effect of these compounds on the respiratory behaviour of the tissue, have been investigated. Thirdly, direct evidence for the metabolic destruction of certain amino acids was sought by using [^{14}C]amino acids, and collecting the $^{14}CO_2$ liberated during the period of uptake.

MATERIALS AND METHODS

Materials

The amino acids used in these and all subsequent experiments were commercial samples. DL-[2-^{14}C]- and DL-[3-^{14}C]-phenylalanine, and DL-[1-^{14}C]leucine were obtained from Tracerlab Inc., Boston, Mass., U.S.A. L-[$^{14}C_9$]Phenylalanine was obtained from the Radiochemical Centre, Amersham, Bucks. The specific activities of the amino acids ranged from 0·3 to 1·1 mc/m-mole.

Methods of estimation

(i) Amino acids recovered from the tissue after uptake were estimated by the method of quantitative paper chromatography, described by Connell, Dixon & Hanes (1955). The following solvents and times of development were used with a descending system.

Propanol–water (4:1, v/v), 18 hr. for alanine; butanol–acetic acid–water (4:1:1, by vol.), (*a*) 7 hr. for leucine, isoleucine, phenylalanine, valine, methionine; (*b*) 36 hr. for glutamic and aspartic acid, histidine, ornithine, lysine (with a pad clipped to the bottom of the paper).

(ii) The disappearance of amino acids from the incubating media in uptake experiments was measured by direct development of the colour, without prior chromatographic separation, in samples (volume not exceeding 1 ml.) of the solution with 2 ml. of the ninhydrin reagent of Connell *et al.* (1955). The accuracy of this procedure was such that recoveries of 100±2% were obtained.

The amount of ^{14}C taken up from solutions of labelled amino acids was calculated from the values for percentage disappearance of the amino acids, and the activity of the original solution determined directly by counting a sample of the [^{14}C]amino acid solution dried on a planchet. This procedure was adopted because of the greater accuracy and ease of the ninhydrin method of estimation, and had the advantage of avoiding errors due to the presence of ^{14}C-labelled compounds containing no amino nitrogen.

Tissue

The treatment of the tissue for use in recovery experiments did not differ from that described previously (Birt & Hird, 1956). However, for experiments with the Warburg apparatus it was found more convenient to prepare disks (0·5 cm. diam.) in place of the larger slices used in other experiments. The tissue was cut into slices (thickness 1 mm.) from which the disks were removed with a stainless-steel cork borer. The disks were washed in the usual way; when required for an experiment they were blotted lightly between filter paper before being placed in the Warburg flasks.

Experimental procedure

(*a*) *Recovery experiments.* Slices of tissue (2·5 g. fresh wt.) were incubated at 25° in a 50 ml. conical flask containing a solution (16 ml.) of the amino acid (5 mM). The flask was shaken at 150 oscillations/min. for a period of 6–7 hr.; at the end of this time, a sample (15 ml.) of the incubating solution was removed and made up to 25 ml. This is referred to as the 'final' solution. The variation of 1 hr. in the time of incubation for different amino acids was not easily avoidable; but, as no comparisons involving a standard time of uptake were made, this was not important. The tissue plus the residual incubating solution (1 ml.) still in the original conical flask was left standing overnight, at 5°, after addition of ethanol (9 ml.); after mixing, 8 ml. of the liquid was removed and placed in a 50 ml. standard flask. Next, the tissue was extracted five times with 70% (v/v) ethanol (9 ml.) with continual shaking at 40° for 0·5 hr.; 8 ml. of each successive extract was withdrawn. Preliminary experiments with L-histidine had shown that this procedure gave complete extraction; for example, successive extracts of a sample of tissue which had taken up 54 μmoles of L-histidine yielded 28, 13, 4, 2, 3, 2 and 0 μmoles of amino acid. The six extracts were combined and made up to 50 ml.

As a measure of the original concentration of the amino acid, a volume (15 ml.) of the amino acid equal to that used for uptake in the recovery experiment was made up to 25 ml. This is termed the 'original' solution.

The entire process was repeated for tissue incubated in solutions of each of the amino acids used, and for a control tissue in water.

For each amino acid, equal volumes of the 'original' and 'final' solutions and of the pooled tissue extracts were combined thus:

(i) 'Original' solution plus extract of tissue incubated in water (to estimate total amino acid present in the solution before incubation, together with that in tissue which had not taken up amino acid).

(ii) 'Final' solution plus extract of tissue incubated in amino acid solution (to estimate amino acid present finally in solution and tissue after uptake).

Samples of these mixtures were analysed in two ways:

(1) Total ninhydrin-positive material was measured by direct estimation with the ninhydrin reagent.

(2) After chromatography, the recovery of the amino acid taken up by the tissue was determined. An allowance was made for the amino acid in the incubating solution (1 ml.) that remained in contact with the tissue at the end of the experiment.

The amino acid taken up by the tissue was determined directly from the ninhydrin-colour densities given by the 'original' and 'final' solutions.

It was found that appreciable amounts of lysine and ornithine remained adsorbed on the tissue at the end of the extraction procedure; however, more than 90% of similar amounts of these amino acids adsorbed on an equivalent sample of tissue was released by two further extractions with Na_2CO_3 (10%, w/v; anhydrous salt). After adjustment of pH, these solutions were analysed directly with the ninhydrin reagent.

(*b*) *Measurement of oxygen consumption.* The conventional Warburg apparatus was used. The main compartment contained 3 ml. of the amino acid solution or water, and 30–40 disks of tissue. The 'fluid' volume in the main compartment was determined by adding a similar number of disks to water in a measuring cylinder. The volume of the disks was always close to 0·5 ml., and this volume was included in the determination of the constant for the apparatus. N-NaOH (0·15 ml.) was used in the centre well. The bath temperature was 25°, and the manometers were shaken at 100 oscillations/min. Air was used as the gas phase. The first reading was taken 15 min. after the taps were closed. In experiments of long duration it was necessary to reset the manometers. At the end of the experiment, samples of the solution were withdrawn for amino acid estimation; the disks were removed, blotted lightly and the fresh weight was determined.

(*c*) *Collection and measurement of* $^{14}CO_2$. Collection of $^{14}CO_2$ was made in the Warburg apparatus; the initial procedure was the same as that described above. At the end of the experimental period, the filter-paper roll, which had been inserted in the centre well, and washings from this well were transferred to a stoppered glass vessel. When CO_2 was collected in successive periods during the experiment, a new roll of filter paper was placed in the same vessel, the apparatus re-assembled and the experiment continued. The CO_2 was released from the alkali by acidification and collected in a saturated solution of baryta; the entire process was carried out in an all-glass apparatus, which was evacuated before the release of the CO_2. The $BaCO_3$ was spun down in a centrifuge, the excess of baryta decanted and the precipitate washed twice with water and once with acetone. It was then suspended in ethanol (90%), plated out in a weighed planchet and dried by standing for some hours in a desiccator containing $CaCl_2$; this was followed by heating with an infrared lamp. The planchet was reweighed and the weight of $BaCO_3$ (10–20 mg.) calculated to permit a correction for self-absorption. Counting was done in a gas-flow counter (Tracerlab SC-16 windowless flow counter; Tracerlab autoscaler).

RESULTS

Recovery of amino acids after uptake by the tissue. It had already been established that histidine, as measured by the imidazole-colour reaction, was fully recoverable from the tissue after uptake (Birt & Hird, 1956). However, it was desirable to repeat this determination by a method specific for the intact amino acid. Accordingly, an experiment was performed in which the recovery of not only L- and D-histidine, but also of a number of other amino acids, was measured. Results are presented in Table 1. It is clear that metabolism of up to

Table 1. *Recovery of amino acids after uptake by carrot slices*

Tissue was washed for 140 hr. before use; 2·5 g. fresh wt. (water content 2·25 g.) was incubated in each of the appropriate solutions (16 ml.; amino acids 5 mM). Initial pH, 5·0; duration of uptake was 6–7 hr.

Amino acid	Total ninhydrin-positive material recovered (%)	Uptake (μmoles)	Recovered from tissue* (μmoles)	Recovery after uptake† (%)	Internal concn. / External concn.
L-Glutamic acid	86	33·5	9·5	28	1·5
L-Aspartic acid	84	33·0	10·5	32	1·6
L-Alanine	88	25·0	6·8	27	0·9
D-Alanine	100	22·5	20·0	89	2·5
L-Phenylalanine	97	28·0	15·5	55	2·1
D-Phenylalanine	98	28·0	25·0	89	3·4
L-Leucine	103	25·5	21·5	84	2·8
D-Leucine	98	17·5	17·0	97	1·9
L-Isoleucine	104	21·5	18·5	86	2·3
D-Isoleucine	99	20·0	18·2	91	2·2
L-Valine	101	28·0	24·5	87	3·4
D-Valine	100	20·5	19·5	95	2·3
L-Methionine	103	20·0	16·8	84	2·0
D-Methionine	101	25·0	24·3	97	3·2
L-Histidine	100	50·5	49·5	98	12·2
D-Histidine	100	40·0	37·0	93	6·6
L-Lysine	97	32·0	29·0 (23·0)‡	91	4·3
L-Ornithine	98	25·5	23·5 (18·5)‡	92	3·0

* Tissue blank for total ninhydrin-positive material (after incubation) equivalent to 17 μmoles/2·5 g. fresh wt.

† Variation in individual estimations was ±2·5%; as the final results are determined by difference, the variation in these will be of the order of ±15%.

‡ Figures in parentheses refer to recoveries obtained before the use of sodium carbonate as extractant; for details see text.

10% could not be detected with certainty; however, it can be seen that: (*a*) Within the limits of experimental accuracy the D-amino acids were fully recoverable. (*b*) With the exception of glutamic and aspartic acid, and alanine and phenylalanine, there was little metabolic destruction of the amino acids used. (*c*) As the total ninhydrin-positive material (which includes ammonia) is substantially higher than the corresponding recovery of the amino acids which are metabolized, it would seem that there is evidence for their transamination or deamination, or both. (*d*) All the amino acids except L-alanine were accumulated against a concentration gradient.

Oxygen consumption of tissue during amino acid uptake. When the oxygen consumption of tissue exposed to solutions of amino acids was measured, it was found that tissue washed for more than about 20 hr. before use showed a greater oxygen consumption in the presence of the amino acid, with the exception of one sample incubated in D-phenylalanine (Table 5). In all experiments of this kind, the rate of oxygen uptake, both in amino acid solution and water, did not diminish during the experimental period (Figs. 1 and 2, Table 2). Tissue whose oxygen consumption was enhanced by an amino acid was affected similarly by potassium chloride (Fig. 2), a salt shown by other workers to increase the respiration of washed carrot slices (see Sutcliffe, 1953). Sometimes, though not invariably, tissue washed for 20 hr. or less, while accumulating amino acid from the solution, did not exhibit an increased oxygen consumption (Table 3). In experiments with L-phenylalanine, L-leucine and glycine with longer periods of washing, it was found that the stimulation of respiration increased with increasing concentration of the amino acid but flattened off or fell at high concentrations. Results of experiments of this kind are given for phenylalanine in Tables 3 and 4. Similar results have been obtained with glycine (Table 2) and with L-leucine.

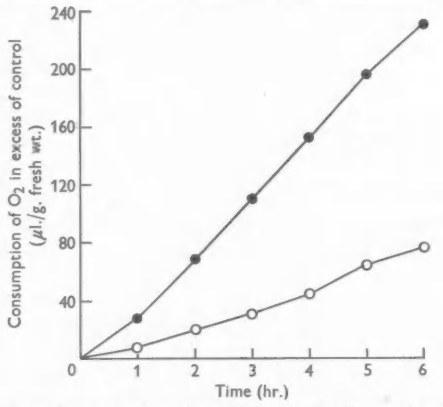

Fig. 1. Oxygen consumption of tissue during the uptake of L- and D-phenylalanine: ●, L-phenylalanine; ○, D-phenylalanine. Tissue (40 disks) was washed for 70 hr. before use, then incubated in 3 ml. of the appropriate solution (5 mM) in the Warburg apparatus.

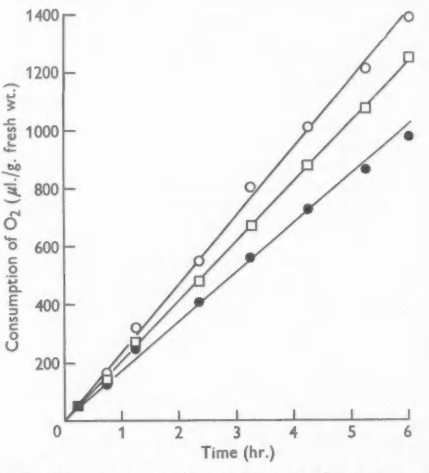

Fig. 2. Oxygen consumption of tissue in the presence of glycine and potassium chloride: ○, potassium chloride; □, glycine; ●, water. Tissue (40 disks) was washed for 60 hr. before use, then incubated in 3 ml. of the appropriate solution (20 mM) in the Warburg apparatus.

Table 2. *Effect of concentration of glycine in medium on oxygen consumption by carrot slices*

Tissue (40 disks) was washed for 25 hr. before use, then incubated in 3 ml. of the appropriate solution in the Warburg apparatus. Duration of uptake was 6 hr.

Concn. of amino acid (mM)	Oxygen consumption (μl./g. fresh wt.)				Oxygen consumption over 6 hr. (% of control)
	0–1 hr.	1–2 hr.	2–4 hr.	4–6 hr.	
0·0	135	144	230	273	100
0·5	142	135	291	292	110
5·0	144	152	308	340	121
10·0	153	149	317	310	119
25·0	140	156	300	324	118

Table 3. *Effect of concentration of* L-*phenylalanine on oxygen consumption by carrot slices*

Tissue (40 disks) was incubated in 3 ml. of the appropriate solution in the Warburg apparatus. Duration of uptake was 6 hr.

Duration of washing (hr.)	Initial concn. (mM)	Oxygen consumption (μl./g. fresh wt./6 hr.)	Uptake of amino acid (μmoles/g. fresh wt./6 hr.)	Oxygen uptake (% of control)
20	0·0	903	—	100
20	0·5	915	—	101
20	5·0	917	16·0	101
20	25·0	909	—	101
20	50·0	915	42·0	101
168	0·0	1080	—	100
168	0·5	1160	—	107
168	5·0	1240	18·0	115
168	25·0	1290	—	119
168	50·0	1220	—	113

Table 4. *Effect of concentration of* L-*phenylalanine on its uptake and on oxygen consumption by carrot slices*

Tissue (40 disks) was incubated in 3 ml. of the appropriate solution in the Warburg apparatus. Duration of uptake was 6 hr.

Duration of washing (hr.)	Initial concn. (mM)	Oxygen consumption (μl./g. fresh wt./6 hr.)	Uptake of amino acid (μmoles/g. fresh wt./6 hr.)	Oxygen consumption (% of control)
20	0·0	893	—	100
20	0·5	1125	3·1	126
20	5·0	1014	17·0	113
20	25·0	1101	31·0	124
20	50·0	996	42·0	112
83	0·0	1110	—	100
83	0·5	1212	3·0	109
83	5·0	1220	22·5	110
83	25·0	1319	44·0	119
83	50·0	1208	44·0	109
190	0·0	1531	—	100
190	0·5	1794	—	117
190	5·0	1936	10·5	126
190	25·0	1888	29·0	123
190	50·0	1939	—	126

Stimulation of oxygen uptake has been obtained with both the L- and D-isomers of phenylalanine and leucine, though it did not always occur with D-phenylalanine (Table 5; however, see also Fig. 1). It can be seen from Table 5 that the oxygen consumption in the presence of L-phenylalanine was usually greater than that in the presence of L- and D-leucine and D-phenylalanine. It has already been shown that the metabolism of L-phenylalanine by the tissue is greater than that of these other amino acids, and it was thought that this might be the reason for the observed differences in oxygen consumption. Accordingly, oxygen consumption in the presence of amino acids subject to considerable metabolic destruction was compared with that in the presence of amino acids which are not metabolized to the same extent by this tissue. The results, presented in Table 6, showed that the increase in oxygen consumption by the tissue is most marked in the presence of aspartic and glutamic acids, less so with L-alanine and L-phenylalanine. The greatest stimulation, calculated per molecule of amino acid taken up, was obtained with aspartic acid. Thus the substantial metabolic destruction of certain amino acids previously described (Table 1) is accompanied by a considerable increase in the oxygen consumption of the tissue.

Production of $^{14}CO_2$ *during the uptake of* [^{14}C]-*amino acids.* The appearance of radioactivity in the carbon dioxide collected in successive periods during an experiment with L-[^{14}C]phenylalanine and DL-[1-^{14}C]leucine is shown in Table 7. It can be seen that whereas there was some progressive increase in the consumption of oxygen by each batch of tissue exposed to L-phenylalanine, there was a much greater progressive increase in the activity of the carbon dioxide collected per hour during the course of the experiment; this difference was not found with DL-leucine. The final column can be

taken as a measure of the percentage destruction of the amino acid taken up. As the phenylalanine was uniformly labelled, there is an uncertainty in relating the $^{14}CO_2$ produced to the percentage metabolism. The minimum value for this percentage is given in the final column of Table 7, i.e. 2·3% of the amino acid taken up. The maximum value (assuming decarboxylation of C-1 only) would be $9 \times 2{\cdot}3$, i.e. 20·7%. With DL-[1-^{14}C]-leucine, on the other hand, all of the $^{14}CO_2$ produced was drawn from C-1 of the amino acid. However, as it is probable that only the L-isomer is metabolized (see Table 1), the maximum value for metabolism of C-1 would be about 12%. The maximum value is calculated on the assumption of an equal uptake of both isomers from solution.

Thus it would seem that these two amino acids, after uptake by the tissue, are drawn slowly into metabolic processes which lead to decarboxylation reactions; the total amount disappearing appeared to be greater for L-phenylalanine than for L-leucine (Table 7), a result in agreement with those obtained from experiments in which recovery and oxygen consumption were measured.

Saturation of the processes responsible for carbon dioxide production was shown in an experiment with DL-[1-^{14}C]leucine. It can be seen from Table 8 that, although the activity taken up in-

Table 5. *Oxygen consumption by carrot slices in the presence of* D- *and* L-*isomers of leucine and phenylalanine*

Tissue (40 disks) was incubated in 3 ml. of the appropriate solution in the Warburg apparatus; a separate batch of tissue was used for each experiment. All tissue samples were washed for at least 24 hr. before use, except in Expt. 2 where the duration of washing was 20 hr. Duration of uptake was 6 hr.

Amino acid	Initial concn. (mM)	Oxygen consumption over 6 hr. (% of control)				
		Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
L-Phenylalanine	5	116	127	126	126	113
D-Phenylalanine	5	98	96	109	—	—
L-Leucine	5	105	104	109	112	110
D-Leucine	5	107	106	110	—	—
L-Phenylalanine	25	139	121	124	123	124
D-Phenylalanine	25	97	96	112	—	—
L-Leucine	25	120	107	114	120	125
D-Leucine	25	115	107	119	—	—

Table 6. *Oxygen consumption by carrot slices in the presence of amino acids*

Tissue (40 disks) was incubated in 3 ml. of the appropriate solution in the Warburg apparatus; experiments marked with an asterisk were with a separate batch of tissue. Duration of uptake was 6 hr.

Amino acid	Duration of washing of tissue (hr.)	Oxygen consumption (% of control consumption)	Excess of oxygen consumption (μmoles) / Amino acid taken up (μmoles)
Glutamic acid	24	169	4·0
	280*	197	2·4
	350	175	6·0
Aspartic acid	24	164	5·5
	280*	175	4·4
	350	155	12·0
L-Phenylalanine	24	129	0·8
	280*	147	0·9
	350	116	0·9
D-Phenylalanine	24	104	0·1
	280*	143	0·6
	350	116	0·8
L-Alanine	24	123	0·6
	280*	175	1·3
	350	126	1·5
D-Alanine	24	124	0·7
	280*	135	0·9
	350	111	0·5
L-Histidine	24	125	0·4
	280*	158	1·0
	350	114	0·5

creases by about 70% as the external concentration of the amino acid is raised from mM to 5 mM, there is an increase of only about 6% in the activity of the carbon dioxide produced. Further, as the amount of the amino acid taken up decreases, the percentage destruction increases.

Uptake and metabolism of D- *and* L-*isomers of leucine and phenylalanine.* It can be seen from Table 9 that dilution of either DL-[1-^{14}C]leucine or L-[$^{14}C_9$]phenylalanine with the corresponding non-radioactive L-isomer causes a relatively greater reduction in the activity of the carbon dioxide produced than does an identical dilution with the D-isomer. Two factors may operate to produce this result.

(i) If the L-isomer were metabolized more readily than the D-, such a change in activity would result from dilution with the non-radioactive L-amino acid.

(ii) If the D-isomer of DL- mixture were metabolized to produce $^{14}CO_2$, then any inhibition of the uptake of the D-isomer by the L-isomer would reduce the amount of $^{14}CO_2$ liberated. It will be shown subsequently that such inhibition could, in fact, be expected.

Results presented in Table 9 therefore do not necessarily reflect the relative rates of destruction of the isomers taken up by the tissue, for insufficient evidence was obtained in the experiments to permit a clear distinction of the importance of these two factors. For reliable interpretation of these points it would be necessary to use both [^{14}C]isomers separately. However, the recovery experiments (Table 1) show that, within the limits of experimental error, there is no metabolism of D-isomers; this supports the first interpretation.

Metabolism of phenylalanine. Further information about the metabolism of phenylalanine was obtained by using DL-[2-^{14}C]phenylalanine and DL-[3-^{14}C]phenylalanine in addition to L-[$^{14}C_9$]-phenylalanine. Results of an experiment with these three compounds are given in Table 10. It can be seen that all three carbon atoms appear in the carbon dioxide produced; and that the percentage destruction of the amino acid increases with increasing duration of washing of the tissue before use. Assuming that D-phenylalanine does not give rise to $^{14}CO_2$, the following additional calculations may be made with the data given. If the uniformly labelled phenylalanine were subject to the removal of one carbon atom only, the percentage destruction of this type in the tissue washed for 280 hr. would be $(9 \times 3{\cdot}9)$%, i.e. approximately 36%. However, both C-2 and C-3 also give rise to $^{14}CO_2$ (5·2% C-2, 1·2% C-3). Therefore the maximum percentage decarboxylation of C-1 would be $(36 - 5 - 1)$%, i.e. 30%. With tissue washed for only 20 hr., the percentage decarboxylation would be of the order of only 10%.

Table 7. *Metabolism of* [$^{14}C_9$]*phenylalanine and* [1-^{14}C]*leucine by carrot slices*

Tissue was washed for 40 hr. before use, then samples of 40 disks were transferred to the Warburg apparatus and incubated in 3 ml. of the appropriate solution. Amino acid solutions were initially 5 mM; uptake of phenylalanine was 46%, of leucine, 40%. Duration of uptake was 6 hr.

Expt. no.	Amino acid	Oxygen consumption (µl./g. fresh wt.)			$10^{-3} \times$ $^{14}CO_2$ recovered (counts/min./g. fresh wt.)				Total counts/min./g. fresh wt. recovered in $^{14}CO_2$ (% of uptake)
		0–1 hr.	1–2 hr.	2–3 hr.	3–6 hr.	0–1 hr.	1–2 hr.	2–3 hr.	3–6 hr.	Total	
1	Control	145	150	161	488	—	—	—	—	—	—
	L-Phenylalanine	164	171	198	608	—	—	—	—	—	—
	L-[$^{14}C_9$]Phenylalanine	174	178	193	615	1·8	2·2	3·6	24·9	32·5	2·3*
2	Control	196	198	189	587	—	—	—	—	—	—
	DL-Leucine	208	230	222	760	—	—	—	—	—	—
	DL-[1-^{14}C]Leucine	217	228	230	795	6·5	6·2	8·1	24·9	46·0	5·9†

* Assuming that C-1 only is producing carbon dioxide, this figure becomes $9 \times 2{\cdot}3 = 20{\cdot}7$%.
† Calculated on the basis of metabolism of the L-isomer only, this figure becomes about 12%.

As is shown in Table 10, there was an increase in the metabolism of phenylalanine by tissue that had been washed for longer periods of time before use. This suggested the possibility that the altered metabolic behaviour of the tissue towards phenylalanine was due to a fall in the nutritional reserve of the tissue. (In the experimental procedure used, no exogenous substrate apart from the amino acid was added.) Accordingly, the effect of added glucose on the production of $^{14}CO_2$ from [$^{14}C_9$]-phenylalanine, and the effect of added phenylalanine on the production of $^{14}CO_2$ from [$^{14}C_6$]-glucose, was studied, and the results are presented in Table 11. It can be seen that the increases in oxygen consumption produced by glucose and phenylalanine are additive and that each substance interferes with the metabolism of the other. However, under the experimental conditions used, the overall oxygen consumption is comprised of at least two factors, namely, the ground respiration

Table 8. *Metabolism of* DL-[^{14}C]*leucine by carrot slices*

Tissue was washed for 44 hr. before use, then samples of 40 disks were transferred to the Warburg apparatus and incubated in 3 ml. of the appropriate solution. Duration of uptake was 6 hr.

Concn. of amino acid (mM)	Oxygen consumption (μl./g. fresh wt./6 hr.)	$10^{-4} \times$ ^{14}C added (counts/min./g. fresh wt.)	$10^{-4} \times$ ^{14}C taken up from solution (counts/min./g. fresh wt.)	$10^{-4} \times$ $^{14}CO_2$ recovered (counts/min./g. fresh wt.)	$^{14}CO_2$ recovered (% of ^{14}C taken up from solution)
Control	960	—	—	—	—
5·0	1080	270	76	3·5	4·6
1·0	980	53	44	3·3	7·5
0·5	960	27	23	2·8	12·2
0·1	950	5·8	3·1	0·91	29·4
0·05	960	2·9	—	0·47	—
0·01	950	0·57	—	0·083	—

Table 9. *Metabolism of leucine and phenylalanine by carrot slices*

Tissue was washed for (*a*) 20 hr., (*b*) 72 hr., (*c*) 20 hr., before use, then samples of 40 disks were transferred to the Warburg apparatus and incubated in the appropriate solution (3 ml.). Labelled amino acids were DL-[1-^{14}C]leucine and L-[$^{14}C_9$]phenylalanine. Concentration of L-[$^{14}C_9$]phenylalanine was initially 2·5 mM in Expts. (*b*) and (*c*). Duration of uptake was 6 hr.

Amino acid	Ratio of isomers (L:D)	Total concn. (L- + D-) (mM)	$10^{-6} \times$ ^{14}C added (counts/min./g. fresh wt.)	$10^{-4} \times$ $^{14}CO_2$ recovered (counts/min./g. fresh wt.)
(*a*) Leucine	1:4	5	$1{\cdot}2_2$	0·95
	4:1	5	$1{\cdot}1_9$	0·34
	1:4	25	5·6	$2{\cdot}5_3$
	4:1	25	6·4	0·98
(*b*) Phenylalanine	1:0	12·5	2·8	0·05
	1:4	12·5	2·8	$1{\cdot}6_2$
(*c*) Phenylalanine	1:0	2·5	$1{\cdot}6_6$	1·2
	1:1	5·0	$1{\cdot}6_6$	$1{\cdot}0_5$

Table 10. *Metabolism of phenylalanine by carrot slices*

Tissue (40 disks) was incubated in 3 ml. of the appropriate solution in the Warburg apparatus. Amino acid solutions were initially 5 mM. Duration of uptake was 6 hr.

Amino acid	Duration of washing (hr.)	$10^{-6} \times$ ^{14}C added (counts/min./g. fresh wt.)	$10^{-5} \times$ ^{14}C taken up from solution (counts/min./g. fresh wt.)	$10^{-3} \times$ $^{14}CO_2$ recovered (counts/min./g. fresh wt.)	$^{14}CO_2$ recovered (% of ^{14}C taken up from solution)
L-[$^{14}C_9$]Phenylalanine plus D-phenylalanine (unlabelled)	20	$1{\cdot}6_6$	$7{\cdot}6_5$	10_5	1·4
DL-[2-^{14}C]Phenylalanine	11	9·3	3·4	3·9	$1{\cdot}1_5$
DL-[3-^{14}C]Phenylalanine	11	$1{\cdot}4_7$	6·2	$0{\cdot}14_3$	$0{\cdot}0_2$
L-[$^{14}C_9$]Phenylalanine plus D-phenylalanine (unlabelled)	280	$1{\cdot}5_8$	8·4	33	3·9
DL-[2-^{14}C]Phenylalanine	280	9·0	4·3	11	2·6
DL-[3-^{14}C]Phenylalanine	280	$1{\cdot}3_1$	6·3	3·8	0·6

Table 11. *Metabolism of phenylalanine and glucose by carrot slices*

Tissue was washed for 260 hr. before use, then samples of 40 disks were transferred to the Warburg apparatus and incubated in 3 ml. of the appropriate solution. Both amino acid and glucose initially were 5 mM. Duration of uptake was 6 hr.

System	Oxygen consumption (μl./g. fresh wt./6 hr.)	Amino acid uptake (μmoles/g. fresh wt./ 6 hr.)	$10^{-6} \times {}^{14}C$ added (counts/min./g. fresh wt.)	$10^{-5} \times {}^{14}C$ lost from solution (counts/min./g. fresh wt.)	$10^{-4} \times {}^{14}CO_2$ recovered (counts/min./g. fresh wt.)	$^{14}CO_2$ recovered (% of ^{14}C taken up from solution)
L-[$^{14}C_9$]Phenylalanine	1130	15	$1{\cdot}6_6$	6·7	2·6	3·9
L-[$^{14}C_9$]Phenylalanine	1330	12	$1{\cdot}6_1$	5·4	0·97	1·8
[$^{14}C_6$]Glucose	1110	—	—	—	24	—
L-Phenylalanine plus [$^{14}C_6$]glucose	1320	12	—	—	16	—
Control	885	—	—	—	—	—

and the stimulated respiration resulting from the presence of the amino acid; this complexity makes the precise significance of the observation obscure.

DISCUSSION

It has been shown that most of the amino acids studied are taken up by slices of carrot with little or no metabolism; that is, they are accumulated by the tissue. This is true for both D- and L-isomers of the amino acids. L-Glutamic acid, L-aspartic acid, L-alanine and L-phenylalanine, however, undergo considerable metabolic destruction. For the first three of these compounds this result was not surprising; but the considerable metabolism of L-phenylalanine was unexpected. It is of interest that the metabolism of L-phenylalanine increased with the duration of the prior washing. This increase is apparently due to depletion of endogenous reserves of metabolizable substrate as it is decreased when glucose is supplied to the tissue.

The production of $^{14}CO_2$ from DL-[1-^{14}C]leucine, L-[$^{14}C_9$]phenylalanine, DL-[2-^{14}C]phenylalanine and DL-[3-^{14}C]phenylalanine gives clear evidence that C-1 of leucine, and C-1, -2 and -3 of phenylalanine, undergo conversion into CO_2 in carrot tissue. It is of some interest that such metabolism occurs in this tissue; for the removal of C-2 and C-3 of phenylalanine would involve hydroxylation and opening of the benzene ring if the steps involved were the same as those existing in mammalian tissue (Knox, 1955).

Stimulation of oxygen consumption accompanying the uptake of certain organic compounds has already been mentioned. In addition, many workers have shown that the uptake of certain inorganic ions by plant tissue results in an increase in oxygen consumption by the tissue (see reviews by Robertson, 1951; Sutcliffe, 1953; Lundegaardh, 1955; Epstein, 1956). To the present time, no generally acceptable explanation of this behaviour has been given. The results presented in this paper show that the uptake of the amino acids tested is also accompanied by an increase in oxygen consumption. Comparison with the results for recovery experiments shows that this increase is most marked when the amino acid concerned is capable of yielding a keto acid which is readily metabolized; especially is this true of aspartate. With L-phenylalanine and L-leucine, part at least of the increase can be attributed to metabolism, but it is unlikely that the same explanation applies to the increased consumption of oxygen caused by the D-amino acids. While it is possible that the uptake process itself stimulates oxygen consumption, more experimental work is required to clarify this point.

Thus it is clear that, with four of the amino acids tested, disappearance from the external medium cannot be equated with accumulation in the tissue. An assessment of the importance of metabolic destruction of these amino acids would be necessary before a comparison of rates of uptake for these compounds, based solely on disappearance from the external medium, could be made. However, of the large number of amino acids tested, it was only with L-alanine that a concentration gradient in favour of the tissue was not obtained. It follows that carrot cells possess a mechanism which permits the uptake of many amino acids against a concentration gradient. Certain characteristics of this process of uptake are discussed in the next paper.

SUMMARY

1. The metabolic fate of a wide variety of L-amino acids and some D-amino acids, after uptake by slices of carrot, has been investigated.

2. Of the L-amino acids tested, only alanine, aspartic acid, glutamic acid and phenylalanine underwent substantial metabolic destruction. There was no evidence of metabolic destruction of the D-amino acids used. All the amino acids tested, with the exception of L-alanine, were concentrated against a gradient.

3. Under certain conditions the addition of D- and L-amino acids caused an increase in the oxygen consumption of the tissue. With the [^{14}C]amino acids DL-leucine, DL-phenylalanine and L-phenylalanine it has been shown that carrot tissue metabolizes C-1 of leucine and C-1, -2 and -3 of phenylalanine to carbon dioxide. By use of [$^{14}C_6$]glucose and L-[$^{14}C_9$]phenylalanine it has been shown that glucose and L-phenylalanine inhibit the production of carbon dioxide from each other. This effect is more pronounced after prolonged pre-washing of the tissue, which lowers the nutritional reserves of the tissue.

4. The significance of the above observations is discussed.

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Kinetic Aspects of the Uptake of Amino Acids by Carrot Tissue

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In previous investigations, a number of features of the process of uptake of amino acids by carrot tissues have been described (Birt & Hird, 1956, 1958). Thus it has been shown that the D- and L-isomers of many amino acids are taken up against a concentration gradient, and that such uptake is inhibited by certain metabolic inhibitors. Further, it has been shown that the rate of uptake is increased by 'washing' the tissue in water for about 20 hr. or more, and is accompanied by an increased consumption of oxygen by the tissue. The present paper extends certain of the above observations to a wider range of amino acids, and attempts a further characterization of the mechanism of uptake by examination of the kinetics of the system.

MATERIALS AND METHODS

The amino acids used were all commercial samples. All solutions used for incubation, including those containing inhibitors, were unbuffered, but adjusted to pH 5·0. The temperature during incubation was 25°.

Methods of estimation

Histidine was estimated by the method of Macpherson (1946), as modified by Birt & Hird (1956). Other amino acids, including glutamine and asparagine, were estimated with the ninhydrin reagent of Connell, Dixon & Hanes (1955) as described by Birt & Hird (1958).

Tissue

The treatment of the tissue did not differ from that previously described (Birt & Hird, 1956), but experiments were carried out in conical flasks (50 ml.) shaken at 150 oscillations/min. in a water bath at 25° with air as the gas phase. The volume of each experimental solution was initially 20 ml., and the fresh weight of the tissue was 3 g. Uptake of amino acid was followed by withdrawing samples of the incubating solution for analysis at the times indicated. As the water content of the tissue did not alter during the incubation period a change in the concentration in the external medium was used to follow uptake.

RESULTS

Comparison of rates of uptake of amino acids. A comparative study of the rate of uptake of 13 L-amino acids was made. The amino acids chosen were of three types:

(*a*) Those with no net charge on the side chain (e.g. phenylalanine, leucine, glycine).

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(*b*) Those with a net negative charge (glutamic and aspartic acid).

(*c*) Those with a net positive charge (histidine, lysine, ornithine).

In Table 1 the amino acids have been arranged in order of increasing magnitude of uptake over the period 0·5–6·0 hr. These periods represent active uptake plus diffusion and do not include the contribution of adsorption, as this process is completed in the first 30 min. (Birt & Hird, 1956). Though there was some variation in the relative rates for different amino acids with the two samples of tissue from different carrots, it was obvious that the amino acids with no net charge on the side chain were taken up at a greater rate than those with such a charge, i.e. in general, the more lipophilic the molecule, the greater the rate of uptake. The effect of increasing the lipophilic nature of an amino compound was also determined by comparing the rates of uptake of the two acids glutamic and aspartic with those of the corresponding amides. The results, presented in Fig. 1, confirm the generalization made above.

The observation made previously with histidine, i.e. a rapid initial disappearance (0–0·5 hr.) followed by a slower process of active uptake (Birt & Hird, 1956), was found to apply to the uptake of other amino acids with a positive charge on the side chain (arginine, lysine, ornithine). However, the magnitude of the active uptake of histidine is very much greater than that of the other three amino acids. It would seem possible therefore that the p*K* value (6·0) of the imidazole nitrogen of histidine is such that the amino acid behaves more like the neutral amino acids during the process of active uptake, although it is readily adsorbed from the solution at pH 5·0, at which value it possesses a second positive charge. Reference to the strength of the adsorption of lysine and ornithine (p*K* values for the nitrogen of the side chains 10·5 and 10·8 respectively) has been made previously (Birt & Hird, 1958).

The anomalous position of L-methionine, which might be expected to be accumulated at about the same rate as leucine or isoleucine, is of interest; further reference to the unexpected behaviour of this amino acid in the system will be made in the section concerned with competition.

Uptake of D- *and* L-*amino acids.* It has been shown that the uptake of several D-isomers of amino acids is an active process, similar to that operating for L-isomers (see Birt & Hird, 1956). The comparison of rates of uptake of D- and L-isomers was extended to several other amino acids to get further information on this aspect. The results of an experiment of this kind are presented in Table 2. Repetition of the experiment confirmed this result. It can be seen that for several amino acids the uptake of the D-isomer exceeds that of the L-isomer. Table 2 also shows that the rate of uptake

Table 1. *Uptake of amino acids by carrot slices*

Tissue (3 g. fresh wt.) was washed for 22 hr. before use, then incubated in 20 ml. of amino acid solution (5 mM). Initial pH, 5·0.

Amino acid	Uptake (μmoles) 0–0·5 hr.	0·5–6·0 hr.
Expt. A		
Arginine	15	7
Ornithine	12	8
Lysine	12	13
Methionine	5	17
Aspartic acid	6	21
Glutamic acid	4	26
Histidine	15	29
Valine	5	35
Phenylalanine	7	35
Leucine	7	36
Isoleucine	5	38
Alanine	4	44
Glycine	6	44
Expt. B		
Ornithine	24	12
Arginine	22	20
Aspartic acid	2	21
Methionine	7	22
Lysine	18	23
Leucine	6	27
Glutamic acid	5	31
Phenylalanine	12	35
Histidine	19	41
Glycine	12	41
Alanine	8	41
Valine	6	42
isoleucine	6	47

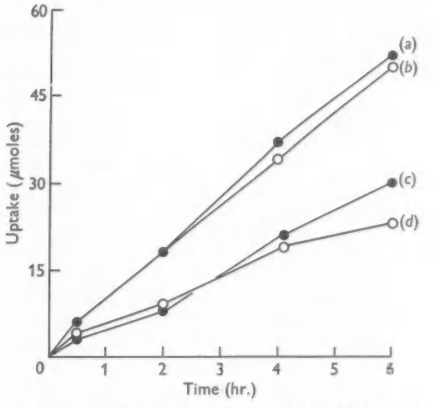

Fig. 1. Uptake of amino acids and their amides: (*a*) glutamine; (*b*) asparagine; (*c*) glutamic acid; (*d*) aspartic acid. Tissue (3 g. fresh wt.) was washed for 24 hr. before use, then incubated in 20 ml. of the appropriate solution (5 mM).

of all the D-amino acids used was approximately linear over a period of 6 hr., i.e. they behaved in the same way as the corresponding L-isomers.

Inhibition of the process of uptake. A first series of experiments, in which amino acids were estimated with chloramine-T, had shown that 2:4-dinitrophenol reduced the uptake of many amino acids, including both D- and L-isomers (Birt & Hird, 1956). Subsequent experiments, in which the estimations were performed with the more sensitive ninhydrin reagent, confirmed this result and made it possible to recognize three types of behaviour in the presence of 2:4-dinitrophenol. These are shown by Figs. 2 (*a*), 2 (*b*) and 2 (*c*).

Table 2. *Uptake of* D- *and* L-*amino acids by carrot slices*

Tissue was washed for 34 hr. before use, then 3 g. fresh wt. was incubated in 20 ml. of the appropriate solution. Initial concentration of all solutions was 5 mM. Duration of uptake was 6 hr.

	Uptake (μmoles)			
Duration of uptake (hr.) ...	0·5	2·0	4·0	6·0
Amino acid				
L-Phenylalanine	3	13	23	38
D-Phenylalanine	4	19	38	55
L-Leucine	7	14	25	38
D-Leucine	3	13	26	43
L-Isoleucine	3	15	32	47
D-Isoleucine	3	11	21	30
L-Alanine	5	14	29	42
D-Alanine	4	16	34	47
L-Tryptophan	4	6	14	22
D-Tryptophan	2	8	13	18
L-Valine	7	18	35	50
D-Valine	4	14	29	40
L-Methionine	5	10	12	16
D-Methionine	3	11	20	29

Table 3. *Inhibition by dinitrophenol of the uptake by carrot slices of amino acids and amides*

Tissue used in experiments marked with an asterisk was from a separate batch; all tissue was washed for 21 hr. before use, then 3 g. fresh wt. was incubated in 20 ml. of appropriate solution. Concentration of all solutions was 5 mM. 2:4-Dinitrophenol (DNP) was 0·1 mM.

		Uptake (μmoles)	
Amino acid	Duration of uptake (hr.)	Control	Control + DNP
Arginine	0·5	23	20
	3·0	44	21
	6·0	48	22
Methionine	0·5	8	2
	3·0	24	4
	6·0	39	4
Glycine	0·5	9	8
	3·0	44	13
	6·0	74	24
*Asparagine	0·5	1	0
	6·0	23	5
Glutamic acid	0·5	3	0
	3·0	24	0
	6·0	43	2
*Glutamine	0·5	6	2
	6·0	52	24

Amino acids with a second positive charge at the pH used, e.g. ornithine, showed an initial adsorption phase not affected by 2:4-dinitrophenol, and a subsequent uptake which was affected. Glutamic and aspartic acids with a second negative charge had a linear curve for uptake from the control solutions, and little or no uptake in the presence of the 2:4-dinitrophenol. Amino acids without a third charge, like leucine, had linear curves of uptake both in the presence and absence of 2:4-dinitrophenol, though the rate in the latter was greatly reduced. Further examples of these three patterns of uptake are given in Table 3.

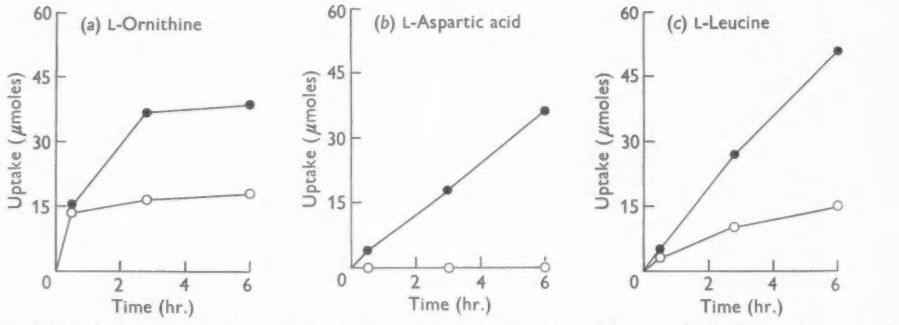

Fig. 2. Effect of 2:4-dinitrophenol on the uptake of amino acids: ●, amino acid; ○, amino acid in the presence of 2:4-dinitrophenol (0·1 mM). Tissue (3 g. fresh wt.) was washed for 22 hr. before use, then incubated in 20 ml. of the appropriate solution (5 mM).

Table 4. *Inhibition of the uptake by carrot slices of* L-*leucine*

Tissue (3 g. fresh wt.) was washed for 120 hr. before use, then incubated in 20 ml. of the appropriate solution. Initial concentrations: leucine, 5 mM; hydrogen cyanide, mM; sodium fluoride, 20 mM; sodium iodoacetate, 5 mM; 2:4-dinitrophenol (DNP), 0·1 mM.

Duration of uptake (hr.)	Uptake (μmoles)				
	Control	Control + cyanide	Control + fluoride	Control + iodoacetate	Control + DNP
0·5	4	2	2	2	4
2·0	12	3	5	2	8
4·0	22	4	7	2	10

Additional information about the necessity for respiratory and glycolytic activity during uptake was obtained by the use of inhibitors other than 2:4-dinitrophenol. The results presented in Table 4 show that the uptake of L-leucine was markedly reduced by hydrogen cyanide and sodium iodoacetate, as well as by 2:4-dinitrophenol. Sodium fluoride (20 mM) has also been shown to inhibit the uptake of leucine (Table 4), though a similar result was not obtained at a concentration of mM.

Saturation of the uptake process. The relationship existing between the rate of active uptake and the initial concentration in the incubating solution was investigated. However, before such an investigation can be made, a correction for inactive processes such as adsorption and diffusion is necessary. In an attempt to make such a correction, two different measurements of inactive uptake have been made: first, in the presence of 2:4-dinitrophenol and secondly with tissue washed for only 0·1 hr. The second method was used because it had been found that slices washed for short periods of time (0·1–0·5 hr.) were incapable of significant active uptake [for example, during incubation for 5 hr. in a solution of L-leucine (0·5 mM), the uptake by slices washed for 0·1 hr. was 4 % of the uptake by a similar quantity of the same tissue washed for 48 hr.], and had the advantage that the tissue was not exposed to any metabolic or respiratory inhibitor. The graphs of Figs. 3 (*a*), 3 (*b*), 3 (*c*) and 3 (*d*) indicate that qualitatively similar results were obtained with the two methods, though neither was entirely satisfactory. However, it is apparent from Fig. 3 that saturation of the overall uptake mechanism occurs below 50 mM for L- and D-leucine and L-histidine, whereas this does not appear to hold for L-lysine and L-aspartic acid.

It has been shown that both aspartic acid and lysine differ, in their behaviour during uptake, from leucine and histidine. Aspartic acid is subject to substantial metabolism, lysine is strongly adsorbed by the tissue slices and the overall process of uptake for both these amino acids is more complex than that of the two isomers of leucine and L-histidine.

Competition experiments. Further information about the nature of the relationship between the amino acids and the mechanism responsible for their uptake was obtained by studying the simultaneous uptake of two amino acids from a single solution. By using the ninhydrin reagent (giving total amino acid) and the histidine method (specific for histidine) on samples of such a mixed solution of amino acids, it was possible to measure the uptake of both histidine and the competing amino acid. Because of the initial adsorption of histidine and lysine, the most accurate assessment of the results was obtained by considering the uptake from 0·5 to 6·0 hr. The percentage uptakes in a number of experiments were calculated and are given in Table 5. From this table it can be seen that the following amino acids markedly inhibited the uptake of L-histidine: L-methionine, L-phenylalanine, L-leucine, L-alanine and L-isoleucine. Inhibition by L-lysine was less pronounced. In turn, with one exception, the uptake of each of these amino acids was inhibited by L-histidine. L-Methionine was the most powerful inhibitor of the uptake of L-histidine, reducing it almost to zero; on the other hand, no reduction of the uptake of L-methionine by L-histidine was observed. Uptake of L-aspartic acid and L-glutamic acid was almost completely inhibited by L-histidine, though L-aspartic acid did not affect the uptake of L-histidine. The inhibition produced by L-glutamic acid was slight (Table 5).

The D-isomers, although being markedly inhibited by L-histidine, did not themselves greatly reduce its uptake.

Similar experiments were performed with a series of D-amino acids and D-histidine. Again, methionine proved to be the most powerful inhibitor, whereas D-leucine, D-phenylalanine and D-isoleucine all inhibited the uptake of D-histidine. However, D-histidine either did not alter the uptake of the competing acid (D-isoleucine, D-phenylalanine) or increased it greatly (D-methionine, D-leucine) (Table 5).

It was noteworthy that, for any particular sample of tissue and an amino acid concentration of 5 mM, the total amount of L-amino acid taken up from all the mixtures used was approximately the

same, with the exception of that for the system containing L-methionine.

From the results of the experiments on competition, it would seem that, among the L-isomers, the order of affinity based on the competition studies is L-methionine > L-phenylalanine > L-alanine > L-histidine > L-leucine, L-isoleucine > L-lysine > L-glutamic and L-aspartic acids. (The affinity for L-histidine may be obtained by comparing the uptake from 10 mM-solution with that from 5 mM-solution.) The affinity of the sites for L-methionine appears to be very much greater than for any other amino acids tested, and probably explains the marked ability of L-methionine to compete with L-histidine. Such high affinity may also be relevant to the unexpected low rate of uptake of L-methionine when compared with other amino acids and its D-isomer.

The affinity of the sites for the D-isomers is much lower than that for L-histidine; and there appears to be some gradation in affinity between the different D-acids themselves. The increased uptake of D-methionine and D-leucine in the presence of D-histidine is of interest, and may be related to the observation that L-histidine will initiate protoplasmic streaming in tissue (Fitting, 1933).

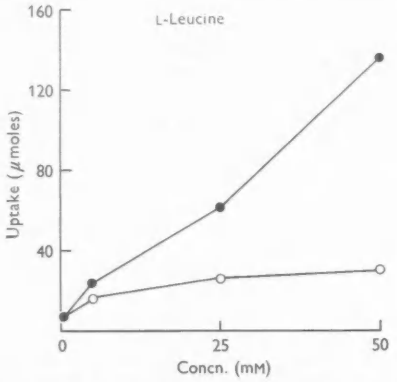

Fig. 3 (*a*). Uptake of L-leucine at four concentrations: ●, uptake of L-leucine; ○, uptake of L-leucine corrected for uptake in the presence of 2:4-dinitrophenol (0·1 mM). Tissue (3 g. fresh wt.) was washed for 48 hr. before use, then incubated in 10 ml. of the appropriate solution. Duration of uptake was 4 hr.

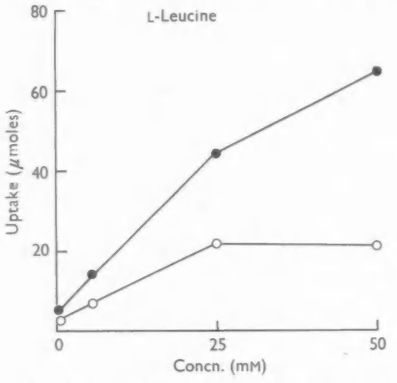

Fig. 3 (*b*). Uptake of L-leucine at four concentrations: ●, uptake of L-leucine by tissue washed for 20 hr. before use; ○, uptake of L-leucine corrected for uptake by tissue washed for 0·1 hr. before use. Tissue (3 g. fresh wt.) was incubated in 10 ml. of the appropriate solution for 4 hr.

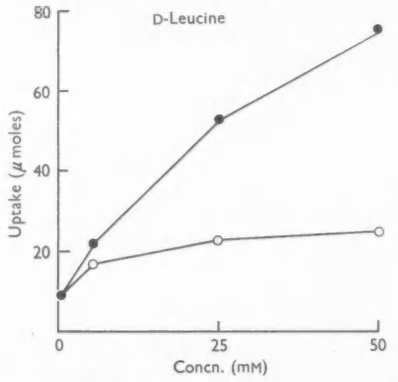

Fig. 3 (*c*). Uptake of D-leucine; conditions were as described for Fig. 3 (*a*). Tissue was washed for 70 hr.

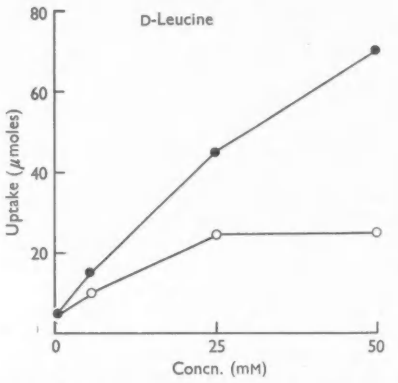

Fig. 3 (*d*). Uptake of D-leucine; conditions were as described for Fig. 3 (*b*). Tissue was washed for 20 hr.

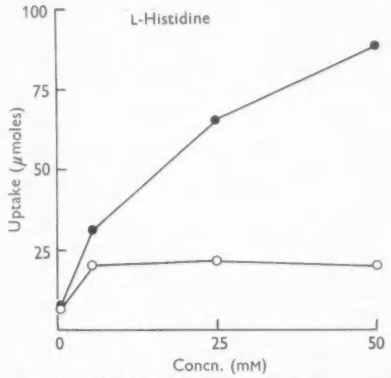

Fig. 3 (*e*). Uptake of L-histidine; conditions were as described for Fig. 3 (*a*). Tissue was washed for 70 hr.

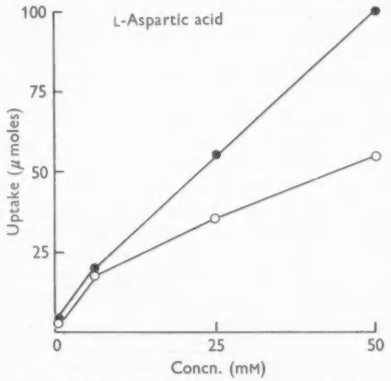

Fig. 3 (*f*). Uptake of L-aspartic acid; conditions were as described for Fig. 3 (*a*). Tissue was washed for 40 hr.

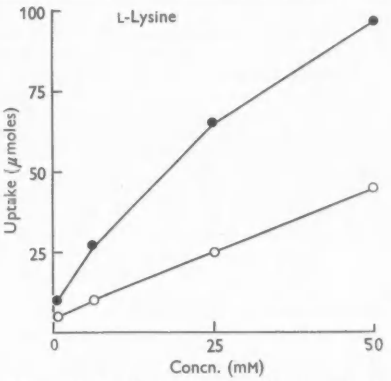

Fig. 3 (*g*). Uptake of L-lysine; conditions were as described for Fig. 3 (*a*). Tissue was washed for 40 hr.

DISCUSSION

The results given in this paper show that the process for uptake of amino acids in carrot tissue possesses certain definable characteristics. The process seems to be common to all the amino acids tested; it has a higher affinity for the L-amino acids, and, in particular, for those with uncharged lipophilic side chains; saturation of the carrier can be achieved with certain of the amino acids tested and energy from glycolysis or respiration or both is necessary for its operation.

A detailed account of the functioning of the mechanism in this plant tissue is not yet possible, but the following considerations may be advanced. It seems possible that the final site for accumulation of amino acids, as of other solutes, is in the vacuole (see Briggs & Robertson, 1957). Therefore there are a number of barriers through which the molecules of amino acids may have to pass during uptake, namely, the phase boundary at the exterior of the cytoplasm, the cytoplasm itself and the boundary between cytoplasm and vacuole—the tonoplast. The penetration of each of these barriers may be a process requiring energy; certainly, movement through the cytoplasm would be aided by cytoplasmic streaming, a process which is known to require metabolic energy (duBuy & Olson, 1940). Any one of these hypothetical, active processes might be the step which becomes limiting when an inhibitor of the uptake of a particular amino acid (e.g. another amino acid or dinitrophenol) is used. With present methods it is not possible to clarify this situation; there may be one or several rate-limiting processes. In addition, apart from the problem of locating the transport mechanism inside the cell, there is the problem of the influence of the intracellular environment on amino acids (whether positively or negatively charged, or with no net charge) as they approach the site of attachment on the carrier system. Thus it is possible to say that there is an active process operating, and to define some of its features, but not to give details of its function or location.

However, in its simplest form the process may involve association with a carrier and movement of the complex, followed by dissociation in the internal environment. There are no precedents for enzymic reactions involving two configurations about an asymmetric centre, and as both D- and L-amino acids are taken up by the same mechanism it is likely that the carrier–amino acid complex does not involve forces of covalent character. Further, the greater affinity of the L-isomers suggests that there may be one point of attachment more than for the D-isomers. The D-isomers of methionine, phenylalanine and leucine have lower affinities for the carrier than their corresponding

Table 5. *Competition between histidine and other amino acids in uptake by carrot slices*

In all experiments, 3 g. fresh wt. of tissue was used after washing for 30 hr. Uptake period was 6 hr.; values used in calculations were those for the uptake in the period 0·5–6·0 hr. Concentration of each amino acid was 5 mM.

Batch of tissue	Competing amino acid	Uptake of L-histidine in the presence of competing acid (% of control uptake)	Uptake of competing acid in the presence of L-histidine (% of control uptake)	Uptake of D-histidine in the presence of competing acid (% of control uptake)	Uptake of competing acid in the presence of D-histidine (% of control uptake)
1	L-Lysine	74, 50, 78	50, —, 67	—	—
	L-Aspartic acid	105, 106, 100	0, 0, 5	—	—
	L-Leucine	47, 40, 59	76, 65, 50	—	—
	D-Leucine	105, 105, 115	0, 0, 29	—	—
2	L-Methionine	7, 0	200, 100	—	—
	L-Glutamic acid	79, 70	25, —	—	—
	L-Phenylalanine	29, 26	50, 48	—	—
	D-Phenylalanine	86, 75	0, 27	—	—
3	L-Alanine	50, 50	72, 90	—	—
	D-Alanine	97, 75	15, 30	—	—
	L-Isoleucine	69, 54	40, 48	—	—
	D-Isoleucine	103, 92	0, 0	—	—
4	D-Isoleucine	—	—	47, 40	125, 120
	D-Phenylalanine	—	—	42, 19	105, 110
	D-Leucine	—	—	21, 19	210, 150
	D-Methionine	—	—	11, 19	185, 135

L-isomers but have greater rates of uptake. This suggests an inverse relationship between affinity as determined by the steric configuration of the molecule and rate, i.e. the relative rate of uptake of stereoisomers is determined largely by the dissociation of the carrier once inside the permeability barrier.

In many of these respects the process exhibits similarities to other transport systems operating in a variety of plant tissues. Thus Brown (1952) has concluded that the uptake of fructose by parenchyma tissue from *Curcurbita* is an active process; and inorganic ions are taken up by an active process which is capable of saturation, and, at least in some tissues, has sites for which various groups of cations and anions compete (see reviews by Epstein, 1956, and Sutcliffe, 1953). Further, amino acids are accumulated in certain animal tissues (Agar, Hird & Sidhu, 1956) by processes with characteristics similar to those found in carrot slices.

SUMMARY

1. Some kinetic aspects of the uptake of a wide variety of amino acids by slices of carrot have been investigated.

2. At the concentration used (5 mM) amino acids with a charge on the side chain are taken up at a slower rate than those without a charge on the side chain. Those amino acids with a positive charge on the side chain as well as being taken up by an active process are strongly absorbed by the tissue. Some D-amino acids are taken up at a faster rate than their L-isomers.

3. The inhibition of the active process of uptake by 2:4-dinitrophenol, cyanide, iodoacetate and fluoride shows that energy is required for the uptake of all the L- and D-amino acids tested.

4. Competition studies suggest that a common carrier system is involved in the uptake of all the amino acids tested. These studies also show that the affinities of the various amino acids for the system differ greatly, those of L-amino acids being greater than those of corresponding D-amino acids and those of amino acids with lipophilic side chains being greater than those of amino acids with hydrophilic side chains.

5. The carrier system involved in uptake can be saturated at high concentration.

6. In relation to the characteristics of the carrier system the significance of the results obtained is discussed.

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The Relationship of Certain Enzymes in Cobra and Rattlesnake Venoms to the Mechanism of Action of these Venoms

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Several authors have reported enzymic activities of cobra venom which they believe to be connected with the toxic action of this venom. In particular, it was reported that the lecithinase activity of cobra venom disrupts mitochondria, thereby changing the spatial relationship of biochemically important enzyme systems (Braganca & Quastel, 1953). A correlation of the toxicity with the ribonuclease activity of various venoms including cobra venom has been reported (Taborda, Williams & Elvehjem, 1952). Chain (1939) found that black tiger-snake venom hydrolyses diphosphopyridine nucleotide. Slotta (1955) and Zeller (1948) suggested that the adenosine triphosphatase of snake venoms is concerned with their toxicity.

Preliminary observations led us to conclude that *Naja flava* (cobra) venom is composed of a relatively simple mixture of proteins which could be separated into a highly toxic fraction and a less toxic fraction. It appeared that such a fractionation might be useful in a study of the relationship of enzyme activity to toxicity. It would be expected that if a given enzyme action of snake venom causes its toxicity, then this enzyme activity should be most marked in the most toxic fraction.

METHODS

Fractionation of Naja flava *venom.* The venom (2 g.) was dissolved in 150 ml. of water in a 600 ml. flask, which was immersed in a mixture of ice and salt, and 300 ml. of 95% (w/v) ethanol at $-5°$ was slowly (15 min.) stirred in. Stirring was continued for a further 15 min. The mixture was then centrifuged; the supernatant solution was separated and kept at $-5°$. The precipitate was dissolved in cold water (25 ml.) and again precipitated by slow addition of cold 95% ethanol (50 ml.) as before. After it had stood for 15 min. the mixture was centrifuged and the supernatant removed, and combined with the previous supernatant and maintained at $-5°$. The precipitate was dissolved in the minimum quantity of water and freeze-dried. With the temperature maintained below freezing, the combined supernatant solutions were evaporated to 25 ml. in a flash evaporator and then freeze-dried. The supernatant (1·6 g.) was designated fraction 1, and the precipitate (0·4 g.) was designated fraction 2.

Electrophoresis. A Spinco model R paper-strip-electrophoresis apparatus was used. All runs were 6 hr. in duration at a constant current of 15 mA. The proteins were stained with bromophenol blue according to the instructions given with the apparatus.

Determination of lecithinase A activity. Lecithinase A activity was determined as follows: Sörensen's phosphate buffer, pH 7·1 (1 ml.), was mixed with 1 ml. of an egg-yolk solution (10 g. of fresh yolk diluted to 1 l.), 2 ml. of a 3% (v/v) suspension of packed human erythrocytes in isotonic NaCl soln. and 1 ml. of an isotonic NaCl soln. containing the venom. This mixture was incubated at 40° for 90 min. The extent of haemolysis of the erythrocytes produced by the lysolecithin was determined by centrifuging the whole erythrocytes from the mixture and reading the density of the clear supernatant solution at 545 mμ. The optical density of the blank (enzyme omitted) was deducted from all readings. When enzyme was present but no substrate, the optical density was indistinguishable from that of the blank.

Determination of succinic dehydrogenase activity. Succinic dehydrogenase activity was measured by a slight modification of the method of Kun & Abood (1949). The following were placed in a test tube: 0·5 ml. of Sörensen's 0·1 M-phosphate buffer, pH 7·4, 1 ml. of the enzyme preparation (a homogenate of rat brain in 6 vol. of buffer), 0·5 ml. of 0·2 M-sodium succinate, 1 ml. of a freshly prepared solution (0·1%) of triphenyltetrazolium chloride and 0·2 ml. of the solution being tested as enzyme inhibitor. This reaction mixture was incubated at 38° for 15 min., then 7 ml. of acetone was added, and the tube stoppered and well shaken and the contents were filtered. Optical-density measurements were made at 480 mμ. The density of the blank solution containing no enzyme was subtracted from the density reading of the experimental determinations. Solutions which contained enzyme but no substrate did not differ significantly from the blank. Inhibition (%) was taken as the corrected density of the reaction mixture with inhibitor, divided by the corrected density of the reaction mixture without inhibitor, multiplied by 100.

Determination of ribonuclease, diphosphopyridine nucleotidase, and adenosine triphosphatase activities. These activities were determined by incubating a mixture of the enzyme (venom), buffer and substrate at 37° and measuring the rate of change in pH produced by the liberation of free phosphoric acid. The buffer used was 0·2 mM-KH_2PO_4–1·8 mM-Na_2HPO_4, pH 7·9. The substrates used were diphosphopyridine nucleotide (2 mg./ml.), adenosine triphosphate (2 mg./ml.), or ribonucleic acid (5 mg./ml.) in isotonic saline. The reaction mixture consisted of 1 ml. of the buffer solution, 1 ml. of substrate and 0·5 ml. of the enzyme (venom) solution in isotonic saline. Measurements of pH were with an electronic pH meter (Photovolt model 110) at 30–60 min. intervals, the solution being returned to the reaction tube after each measurement. The cup assembly

of the pH meter was kept close to incubation temperature with heating tape. When either the substrate or the enzyme was omitted from the reaction mixtures, no pH change was observed. Fig. 1 shows the quantitative response obtained with *Crotalus t. terrificus* venom with adenosine triphosphate as a substrate.

Materials. The snake venoms were obtained from the Miami Serpentarium, Miami, Florida, U.S.A. Diphosphopyridine nucleotide, triphosphopyridine nucleotide and ribonucleic acid were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

RESULTS

Electrophoretic studies and chemical properties of the fraction of cobra venom. Fig. 2 presents the results obtained when cobra venom was subjected to electrophoresis by the paper-strip method. Sodium diethyl barbiturate buffer, pH 8·6, *I* 0·06, was used. The amount of material (venom, etc.) applied in each case was 0·01 ml. of a 10% (w/v) solution. The paper strip obtained indicates the presence of five proteins, labelled *A*, *B*, *C*, *D* and *E* in Fig. 2. Very similar results were obtained when the venom was subjected to electrophoresis with a Sörensen phosphate buffer of pH 6·8, *I* 0·06. Electrophoresis of fraction 1 (see Methods) indicates that it is composed of proteins *A* and *B*, and fraction 2 contains proteins *C*, *D* and *E*. Electrophoretic separation at pH 10 with a glycine–sodium hydroxide buffer, *I* 0·065, revealed that proteins *A* and *B* were still migrating towards the cathode, indicating that the isoelectric point of these proteins is above 10. Included in Fig. 2 is a similar electrophoretic study at pH 8·6 of *C. t. terrificus*. The paper strip obtained reveals what appears to be a single protein migrating toward the anode. Fraction 1, containing proteins *A* and *B*, represents approx. 80% of the total weight of the venom, and fraction 2 makes up 20%. It should be emphasized that the fractionation process is a simple precipitation procedure in which nothing was discarded.

Both fractions were subjected to the Molisch test for carbohydrates: fraction 1 was negative but fraction 2 was positive, indicating the possible presence of glycoprotein.

Toxicity of fractions 1 *and* 2 *of* Naja flava *venom.* The approximate lethal doses (Deichmann & LeBlanc, 1943) of fraction 1 and fraction 2 were respectively 0·33 and 1·5 mg./kg. intravenously in mice, 0·4 and 1·6 mg./kg. subcutaneously in rats and 0·1 and 0·4 mg./kg. subcutaneously in the dog. Thus it is evident that in these animals, by these routes of administration, fraction 1 is about four times as toxic as fraction 2.

The recovery of toxicity obtained in the fractionation procedure based on intravenous administration to mice is shown in Table 1. Since nothing is discarded, total recovery of the toxicity was to be expected unless denaturation occurred. The total 'units' of toxicity (weight divided by the approximate lethal dose in mg./kg.) recovered in the two fractions add up to slightly more than the initial activity, probably a reflexion of the error of the approximate lethal-dose determination. The term toxicity, when used in this instance, means the number of kilograms of mice which would be killed by the intravenous administration of the entire fraction, or the total number of approximate lethal doses in the fraction.

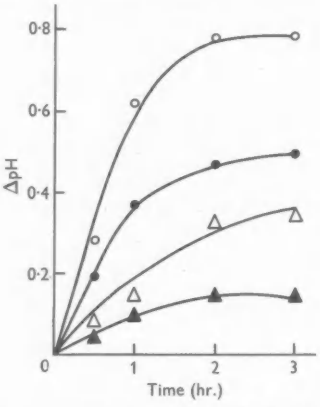

Fig. 1. Quantitative response obtained with *Crotalus t. terrificus* venom with adenosine triphosphate as substrate (substrate concentration was 2 mg. in 2·5 ml.). The highest concentration of venom used (○) was 1·0 ml. of 0·1 mg./ml. of solution (100 μg. in 2·5 ml.), the lowest concentration (▲) was 0·1 ml. of the 0·1 mg./ml. of solution (10 μg. in 2·5 ml.). Intermediate concentrations are: ●, 50 μg. and △, 20 μg./2·5 ml.

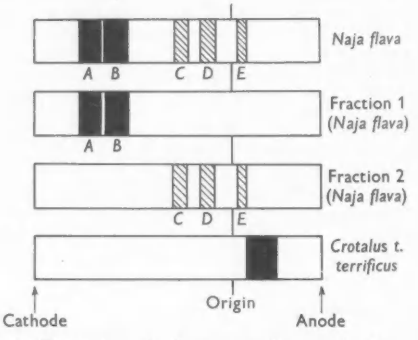

Fig. 2. Electrophoresis of venoms and venom fractions on paper at pH 8·6. For details see text.

Relationship between lecithinase A activity, ability to inhibit succinic dehydrogenase and toxicity. Since we have been able to achieve fractionation of cobra venom into two fractions, one of which is four times as toxic as the other, it was felt that this fractionation could be used to evaluate the relationship between lecithinase A activity, the ability to inhibit succinic dehydrogenase and toxicity. In addition, the effect on lecithinase A potency and the ability to inhibit succinic dehydrogenase of heated snake venoms was investigated. Table 2 shows the lecithinase A potency of the various fractions and whole-venom preparations, and Table 3 shows their ability to inhibit succinic dehydrogenase. Although only about one-half of the lecithinase A activity was accounted for, fraction 2 (the less toxic fraction) is 20 times as potent in lecithinase activity as fraction 1. Fraction 2 is also many times more potent in inhibitory activity against succinic dehydrogenase than fraction 1. The effect of these fractions and venoms was greater if the dehydrogenase was preincubated for 1 hr. with the inhibitors before adding the substrate, which suggests that a direct attack is being made on the enzyme itself. Thus it seems that there is a direct correlation between lecithinase A activity and its inhibitory action against succinic dehydrogenase, but that neither property of this venom is directly related to its toxicity.

Table 1. *Recovery of toxicity in the fractions obtained from* Naja flava *venom*

Toxicity was measured by intravenous injection into mice. A.L.D., approximate lethal dose (Deichmann & LeBlanc, 1943).

	Crude venom	Fraction 1	Fraction 2
Wt. (g.)	2·0	1·6	0·4
A.L.D. (mg./kg.)	0·40	0·33	1·5
Wt./total A.L.D. (kg.)	5000	4850	267

Table 2. *Lecithinase A potency of venoms and venom fractions relative to that of* Crotalus adamanteus *and the effects of heating*

C. adamanteus venom was selected as a standard of reference. Heating was carried out at 100° for 15 min.

Venom	Amount used (μg.)	Wt. of *C. adamanteus* venom with equivalent enzymic activity (μg.)	Potency relative to *C. adamanteus* (%)	Inhibition of potency due to heating (%)
Naja flava	10	6·3	63	—
Fraction 1 (*N. flava*)	50	3·2	6	—
Fraction 2 (*N. flava*)	5	6·3	126	—
Heated (*N. flava*)	10	4·2	42	33
Crotalus t. terrificus	5	7·2	144	—
Heated (*C. t. terrificus*)	10	3·1	31	78
Heated (*C. adamanteus*)	10	4·8	48	52

Table 3. *Inhibition of succinic dehydrogenase*

In the 'immediate' series of experiments, enzyme, inhibitor (venom) and substrate were mixed and immediately incubated at 37° for 15 min. The effect of 'pre-incubation' for 1 hr. of the enzyme and inhibitor before adding the substrate was also investigated.

Venom	Venom added (mg.)	Inhibition (%)	
		Immediate	Pre-incubated for 1 hr.
Naja flava	0·6	0	30
	1·0	29	71
Fraction 1 (*N. flava*)	0·6	0	2
	1·0	0	12
Fraction 2 (*N. flava*)	0·6	87	37
	1·0	68	100
Heated (*N. flava*)	1·0	7	45
Crotalus t. terrificus	0·6	51	91
	1·0	66	100
Heated (*C. t. terrificus*)	1·0	24	48

When these venoms were heated for 15 min. at 100° (the procedure used by Braganca & Quastel, 1953), a consistent moderately destructive effect on both the lecithinase A activity and the inhibition of succinic dehydrogenase was observed. Approximately one-half of the lecithinase A potency and the inhibitory activity against succinic dehydrogenase was destroyed.

Activity of adenosine triphosphatase. Because of the importance of adenosine triphosphate in muscle metabolism and the paralysing effect on muscle of the snake venoms being investigated, the adenosine triphosphatase activity of these venoms and fractions was studied. The results obtained, when the activity of fraction 1, fraction 2 and crude *Naja flava* venom were compared on an equal-weight basis, are shown in Fig. 3. The most potent enzyme activity was obtained with fraction 2, the least with fraction 1, and the crude venom was intermediate in potency. It was considered unlikely that the inherent buffering capacity of the fractions would influence the result when only 50 μg. was used. However, to eliminate this possibility, a series of experiments was performed which showed that heated 50 μg. quantities of one fraction did not influence the change in pH produced by the other.

A similar comparison of the potency of crude *Naja flava* venom with *Crotalus t. terrificus* and *C. adamanteus* venoms was made. The results obtained, in decreasing order of potency, were: *C. t. terrificus*, *C. adamanteus* and *Naja flava*.

Activity of diphosphopyridine nucleotidase. Determination of this activity of the three venoms and the two fractions revealed results similar to the one obtained with adenosine triphosphatase. Again fraction 2 was much more potent than either *N. flava* or fraction 1 (Fig. 3), and again the activity of *Crotalus t. terrificus* was greater than that of *C. adamanteus*, which in turn was more potent than that of *Naja flava*. However, the difference in activity between each of the three whole venoms was relatively slight.

Activity of ribonuclease. Similar results were obtained when the ribonuclease activity of the two fractions was studied. As with the other two enzyme activities, fraction 2 was the most active (Fig. 3). Although the order of potency was the same, there was very little difference in activity between the three whole venoms. The change in pH produced by all of the venoms and fractions with ribonucleic acid was considerably less than the change produced with adenosine triphosphate and diphosphopyridine nucleotide as substrates.

DISCUSSION

The fundamental thesis of these experiments is that if a given venom is separated into a more toxic and less toxic fraction, an enzyme activity of toxicological significance should be present in higher concentration in the more toxic fraction. When several enzyme activities of the two fractions were compared on an equal-weight basis, it was found that the concentration of activity was markedly less in the more toxic fraction. It is barely conceivable that the disproportionate distribution of an inhibitor might explain these results, but it seems more likely that none of the enzyme activities tested offers an explanation for

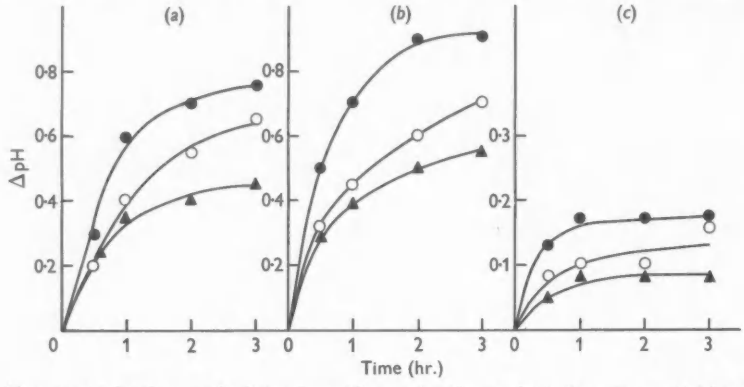

Fig. 3. (*a*) Adenosine triphosphatase, (*b*) diphosphopyridine nucleotidase and (*c*) ribonuclease activities of *Naja flava* venom and fractions 1 and 2 of *N. flava* venom. Each reaction mixture contained 50 μg. of the venom or fraction. The amount of substrate used was 2 mg. of adenosine triphosphate or diphosphopyridine nucleotide and 5 mg. of ribonucleic acid. The total volume of each reaction mixture was 2·5 ml. ○, Crude *N. flava* venom; ▲, fraction 1; ●, fraction 2.

the toxic action of *N. flava* venom. The correlation found between lecithinase A activity and the inhibitory action against succinic dehydrogenase is consistent with the view of Braganca & Quastel (1953) that the lecithinase A action of cobra venom is responsible for the inhibition of succinic dehydrogenase, probably by disruption of the mitochondria. However, our results indicate that both these activities are incidental to the primary toxic action.

The method of preparation by non-denaturing techniques of a fraction (fraction 1) from *N. flava* venom which has most of the toxicity of the venom but little of the enzymic activities is felt to represent an improvement over the method of heating the venom at 100° for 15 min. (Braganca & Quastel, 1953). The fraction obtained is more active, and it seems that the use of a denaturing technique such as heating to simplify a mixture of proteins is biochemically less satisfactory. It is frequently assumed that if a mixture of toxic proteins such as a snake venom is heated and no loss in toxicity is detected, the proteins primarily responsible for the toxicity of the mixture have not been affected by the heat treatment. There is no proof of this assumption. There is no reason to assume that the product obtained by heating cobra venom at 100° for 15 min., whether more, less or equally toxic, is the same biochemically as the original venom. Work is being continued in this Laboratory with this fraction in an attempt to explain its toxicity.

SUMMARY

1. *Naja flava* (cobra) venom has been separated into two electrophoretically distinct fractions, one (fraction 1) four times as toxic as the other (fraction 2).

2. Fraction 1 is composed of two highly mobile, strongly basic proteins comprising 80% by weight of the venom; fraction 2, which makes up the remaining 20% of the venom, consists of three proteins of low mobility. This fraction gives a positive Molisch test for carbohydrate.

3. Fraction 1, which is four times as toxic as fraction 2, is much less potent than fraction 2 with respect to lecithinase A, adenosine triphosphatase, diphosphopyridine nucleotidase and ribonuclease activities, indicating that none of these enzyme activities is of prime importance toxicilogically.

4. Fraction 2 is also a much more potent inhibitor of succinic dehydrogenase than fraction 1. Thus although the lecithinase A activity may well be responsible for the inhibition of succinic dehydrogenase, neither action is of importance as a toxic mechanism.

5. Comparison of the adenosine triphosphatase, diphosphopyridine nucleotidase and ribonuclease activities of *Naja flava* venom with those of two *Crotalus* venoms (*C. t. terrificus* and *C. adamanteus*) revealed that *Naja flava* venom was less active in each case.

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The Effect of Various Metabolites on Incorporation *in vitro* of Labelled Amino Acids into Protein of Normal Rat Diaphragm

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Incorporation of ^{14}C from DL-[1-^{14}C]alanine or from uniformly ^{14}C-labelled L-alanine into protein of the normal rat diaphragm is depressed by addition to the medium of sodium pyruvate and, to a less extent, of glucose (Sinex, MacMullen & Hastings, 1952; Manchester & Young, 1958). Manchester & Young (1958) studied the incorporation of radioactive material from another ten labelled amino acids, but found no diminution of incorporation in the presence of glucose. In the presence of pyruvate, incorporation from several amino acids was slightly smaller, but with only alanine and aspartic acid was the diminution substantial. Sinex *et al.* (1952) considered the possibility that in the presence of pyruvate, either added to the medium or formed from glucose, a

transaminase system (Braunstein & Kritzmann, 1937) could produce unlabelled alanine which might dilute labelled alanine entering the tissue from the medium, and so give rise to a diminution of incorporation into protein of ^{14}C from the added [^{14}C]alanine. If this explanation is correct a smaller incorporation of ^{14}C from glutamic and aspartic acid might occur when glucose or pyruvate is added to the medium, since α-oxoglutarate and oxaloacetate can be formed in the tricarboxylic acid cycle. But a diminution of incorporation might not occur if the keto acids formed are confined to the mitochondrion or are otherwise not accessible to transaminase-enzyme systems.

We have investigated these questions and have sought to elucidate why incorporation of ^{14}C from alanine is much more reduced by addition of glucose or pyruvate than is that of any other amino acid.

MATERIALS AND METHODS

Radioactive materials. Radioactive materials were obtained from the Radiochemical Centre, Amersham, Bucks. In the first experiment, where DL-[1-^{14}C]alanine was incubated in buffer in which diaphragm tissue had previously been shaken, the alanine had a specific activity of about 4·4 mc/m-mole and a concentration of 0·3 μmole/ml. In the experiments with uniformly ^{14}C-labelled glucose (designated [U-^{14}C]glucose) and sodium pyruvate, and with *N*-butyl-*N'*-sulphanilylurea (carbutamide) and *N*-butyl-*N'*-toluene-*p*-sulphonylurea (tolbutamide), the DL-[1-^{14}C]-alanine and the [^{14}C]glucose and pyruvate were all diluted to a specific activity of about 65 μc/m-mole. L-[U-^{14}C]-Alanine, [1-^{14}C]glycine and L-[U-^{14}C]aspartic acid had a specific activity of about 800 μc/m-mole, and L-[U-^{14}C]-leucine of about 400 μc/m-mole. In the experiments with sodium [5-^{14}C]oxoglutarate both the [^{14}C]oxoglutarate and L-[U-^{14}C]glutamic acid had a specific activity of about 400 μc/m-mole, though in other experiments [^{14}C]glutamic acid was used at a specific activity of about 800 μc/m-mole. In experiments with $NaH^{14}CO_3$ about 8 μc was added to each flask. Since 2 ml. of medium contained 54 μmoles of $NaHCO_3$ and 20 ml. of gas phase contained 1 ml. of CO_2 (about 50 μmoles), and a complete distribution of ^{14}C between HCO_3^- ion and CO_2 may be assumed, the specific activity was about 80 μc/m-mole.

Medium. Bicarbonate buffer, pH 7·4 (Gey & Gey, 1936), gassed with O_2+CO_2 (95:5, v/v), was used throughout. Except where otherwise stated, DL-alanine had a final concentration of 2·7 μmoles/ml., L-alanine, glycine, L-leucine, L-glutamic acid and L-aspartic acid 1 μmole/ml. Oxaloacetic acid and α-oxoglutaric acid were dissolved in N-NaOH (1/0·5 mol.prop.) and then diluted with buffer to a final concentration of 12·5 μmoles/ml. Glucose and sodium pyruvate were also used at this concentration. NaCl was added to all media not containing keto acids in such amount as to keep the [Na^+ ion] of the medium constant.

Insulin (crystalline insulin, Boots Pure Drug Co. Ltd., Nottingham) was dissolved in 1·7 mN-HCl to give a concentration of 10 units/ml. A volume (0·1 ml.) of insulin solution (or of 1·7 mN-HCl as control) was added to each flask to make a final concentration of 0·45 unit/ml.

Carbutamide and tolbutamide (Burroughs Wellcome and Co., London) were dissolved in the minimum volume of N-NaOH and diluted with Geys's buffer to a final concentration of 10 mM or mM. Similar quantities of NaOH were added to control solutions and all were gassed to bring the pH to 7·4.

Chromatography. In the experiment where DL-[^{14}C]-alanine was incubated with the fluid in which diaphragm tissue had previously been shaken, 5 μl. samples of the medium were withdrawn at 30 min. intervals and pipetted on to Whatman no. 1 paper. When the spot was dry 5 μl. of 0·4% solution of unlabelled DL-alanine was applied to the same place to act as carrier. Alanine was separated by chromtography with a phenol–ammonia–water system, and was eluted with water from the paper on to a 2 cm. diam. aluminium disk. The solution was dried as a thin film and the radioactivity assessed by the method of Boursnell, Coombs & Rizk (1953). Since the quantities of alanine were very small no correction for self-absorption was required.

Fixation of carbon dioxide. Warburg manometer flasks were used for experiments with $NaH^{14}CO_3$. Each flask contained 2 ml. of Geys's buffer in the centre portion and 8 μc of $NaH^{14}CO_3$ in 0·1 ml. of 0·01 N-NaOH in the side arm. The diaphragm was prepared as before and transferred to Geys's buffer. The side arm was then immersed in a freezing mixture to freeze the solution in the side arm whilst the flask was gassed with O_2+CO_2 or N_2+CO_2 and sealed. The contents of the side arm were melted and added to the diaphragm, and the flasks incubated.

Preparation of diaphragm. A female albino Wistar rat, which weighed 100–150 g. and had fasted 20–24 hr. before use, was decapitated and bled and the diaphragm removed. The two halves of the diaphragm were washed in freshly gassed buffer, and then gently blotted and transferred to small conical flasks containing 2 ml. of medium. As far as possible one half of a diaphragm was used as control for the other half. Two hemidiaphragms were added to each flask. The flasks were gassed, sealed and incubated at 37° in a Dubnoff shaker.

Preparation of protein from diaphragm. After incubation for 2 hr. the diaphragm was removed, and blotted and homogenized in a Potter–Elvehjem homogenizer in 10 ml. of 10% (w/v) trichloroacetic acid. The precipitate was spun down, resuspended in 10 ml. of 10% trichloroacetic acid and heated for 15 min. at 90°. The insoluble material was extracted twice with 1·5 ml. of 0·4 N-NaOH, and the insoluble residue rejected (protein content about 5%). The dissolved protein was reprecipitated by the addition of 5 ml. of 10% trichloroacetic acid. It was washed with 2 ml. of ethanol–ether (50:50, v/v), and then with 2 ml. of ether and was dried in a vacuum desiccator.

Assessment of radioactivity of diaphragm protein. Two hemidiaphragms, combined weight about 200 mg., yielded by the method described 25–30 mg. of protein which had a nitrogen content of about 12·5% (consistent with a protein content of about 80%), and about 0·05% of phosphorus. The samples of protein were ground in a mortar, collected on 1 $cm.^2$ plastic disks, and counted as infinitely thick samples (Popják, 1950), a thin end-window Geiger–Müller tube being used. Sufficient counts were recorded to give a counting error of less than 5%.

Statistical treatment of results. Experiments dealing with [^{14}C]-glucose and -pyruvate and with carbutamide and tol-

butamide were performed in groups of six, one each with and without addition of insulin or carbutamide or tolbutamide in the absence of added substrate, with and without addition of insulin or carbutamide or tolbutamide in the presence of glucose, and with and without addition of insulin or carbutamide or tolbutamide in the presence of pyruvate. Counts found were expressed as a percentage of the counts of the control. This procedure was repeated four or six times. The percentages so obtained were grouped, averaged and the standard errors determined. Significance was assessed on the basis of Student's t test.

In most other experiments incorporation in the presence of various additions to the medium was expressed as a percentage of the control value for incorporation in the absence of these additions. Percentages so obtained were grouped, averaged and the standard errors determined. Significance was assessed on the basis of Student's t test.

RESULTS

Alanine. That the peculiar behaviour of alanine in the presence of glucose or pyruvate (Sinex *et al.* 1952; Manchester & Young, 1958) is not due to destruction of alanine outside the tissue is indicated by the results in Table 1. When [^{14}C]alanine is shaken in a pyruvate-containing medium in which diaphragm tissue has been incubated for 1 hr. and then removed, the amount of alanine does not fall. Such an experiment does not preclude the possibility that the specific activity of the alanine falls because of the appearance of unlabelled alanine in the medium.

Bornstein (1957) observed that carbutamide and tolbutamide *in vitro* inhibited a liver-transaminase system involving alanine and oxoglutarate. If the diminution of incorporation of ^{14}C from alanine into protein of normal diaphragm in the presence of glucose or pyruvate is the result of the dilution of the labelled alanine by unlabelled alanine formed from carbohydrate by transamination (Sinex *et al.* 1952; Manchester & Young, 1958), incorporation of ^{14}C might be stimulated in the presence of carbutamide or tolbutamide. In the presence of these substances formation of alanine from pyruvate would be less than in their absence, and the specific activity of alanine inside the tissue would be higher. However, we have observed that addition of carbutamide and tolbutamide to the medium results in incorporation of ^{14}C from DL-[^{14}C]alanine to a less extent than is observed in the absence of these substances. This was true when glucose or pyruvate was present in the medium, as well as in their absence (Table 2).

On the other hand, when [U-^{14}C]glucose and [1-^{14}C]pyruvate were present in the medium together with DL-[1-^{14}C]alanine, the glucose and pyruvate having the same specific activity as the added DL-alanine, incorporation of ^{14}C was substantially higher in the presence of pyruvate (Table 3). Addition of insulin under these conditions promoted still further the incorporation of ^{14}C into diaphragm protein (Tables 4, 5; Fig. 1).

Table 1. *Effect on* DL-[1-^{14}C]*alanine of incubation in a medium containing pyruvate and in which diaphragm tissue has previously been shaken*

Time of incubation of [^{14}C]alanine (hr.)	Counts/min. from [^{14}C]alanine separated by paper chromatography from 5 μl. of incubating medium	
	Expt. 1	Expt. 2
0	965	986
0·5	931	963
1·0	884	991
1·5	911	913
2·0	—	935
2·5	979	990

Table 2. *Effect of the addition of carbutamide or tolbutamide on the incorporation* in vitro *of* ^{14}C *from* DL-[1-^{14}C]*alanine into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added carbutamide or tolbutamide. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Effect of the addition of	Medium including: No added substrate. Radioactivity in diaphragm protein		Glucose. Radioactivity in diaphragm protein		Pyruvate. Radioactivity in diaphragm protein	
	Control experiments (counts/min./disk)	Percentage of control value in the presence of added carbutamide or tolbutamide	Control experiments (counts/min./disk)	Percentage of control value in the presence of added carbutamide or tolbutamide	Control experiments (counts/min./disk)	Percentage of control value in the presence of added carbutamide or tolbutamide
Carbutamide (10 mM)	93	82·0±4·3 (4)	75	90·0±4·1 (4)	50	87·0±5·2 (4)
Tolbutamide (10 mM)	91	9·1±1·8 (4) [P<0·001]	70	8·2±0·8 (4) [P<0·001]	52	21·6±1·7 (4) [P<0·001]
Tolbutamide (mM)	105	94·0±2·6 (4)	88	99·2±2·2 (4)	59	93·2±1·0 (4)

Effect of oxoglutarate. In view of the results obtained with alanine and the related keto acid, pyruvate, it was of interest to study the effect of addition to the medium of α-oxoglutarate on the incorporation of ^{14}C from amino acids and, in particular, that from glutamic acid. Incorporation of ^{14}C from [^{14}C]glutamic acid was substantially depressed when oxoglutarate was added to the medium (Table 6). Addition of oxoglutarate also diminished, though to a much less extent, incorporation of ^{14}C from [^{14}C]-glycine and -leucine (Table 6). When [5-^{14}C]oxoglutarate was present in the medium together with L-[U-^{14}C]glutamic acid, the oxoglutarate having the same specific activity as the added L-glutamic acid, incorporation of ^{14}C was much higher in the presence of oxoglutarate (Table 7). However, addition of oxoglutarate did not depress significantly incorporation into protein of ^{14}C from either alanine or aspartic acid; on the contrary, under these conditions incorporation of ^{14}C from aspartic acid was somewhat enhanced (Table 6).

Effect of oxaloacetate. As with α-oxoglutarate, addition of oxaloacetate markedly depressed incorporation of ^{14}C into protein from glutamic acid and slightly diminished, though to a much less extent, incorporation of ^{14}C from glycine and leucine (Table 8). Also like oxoglutarate, addition

Table 3. *Effect of the addition of* [^{14}C]*glucose or* [^{14}C]*pyruvate to a medium containing* DL-[1-^{14}C]*alanine on the incorporation* in vitro *of* ^{14}C *into protein of normal rat diaphragm*

No insulin was added to the medium. Results are expressed as a percentage of the control value found in the absence of added glucose or pyruvate. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Radioactivity in diaphragm protein		
Control experiments (counts/min./disk)	Percentge of control value in the presence of DL-[1-^{14}C]alanine and of added	
	[^{14}C]Glucose	[^{14}C]Pyruvate
66	187·8±19·3 (6) [P<0·01]	211·3±14·3 (6) [P<0·001]

Table 4. *Effect of the addition of insulin* (0·5 *unit/ml.*) *to a medium containing* DL-[1-^{14}C]*alanine without added oxidizable substrate or with* [^{14}C]*glucose or* [^{14}C]*pyruvate, on the incorporation* in vitro *of* ^{14}C *into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added insulin. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Medium including					
No added substrate. Radioactivity in diaphragm protein		[^{14}C]Glucose. Radioactivity in diaphragm protein		[^{14}C]Pyruvate. Radioactivity in diaphragm protein	
Control experiments (counts/min./disk)	Percentage of control value in the presence of insulin	Control experiments (counts/min./disk)	Percentage of control value in the presence of insulin	Control experiments (counts/min./disk)	Percentage of control value in the presence of insulin
66	136·2±7·9 (6) [P<0·01]	117	158·3±6·7 (6) [P<0·001]	132	136·3±7·2 (6) [P<0·01]

Table 5. *Effect of the addition of* [^{14}C]*glucose or* [^{14}C]*pyruvate to a medium containing both insulin and* DL-[1-^{14}C]*alanine on the incorporation* in vitro *of* ^{14}C *into protein of normal rat diaphragm*

Insulin (0·5 unit/ml.) was present in each experiment. Results are expressed as a percentage of the control value found in the absence of added glucose or pyruvate. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Radioactivity in diaphragm protein		
Control experiments (counts/min./disk)	Percentage of control value in presence of added	
	[^{14}C]Glucose	[^{14}C]Pyruvate
91	218·7±19·1 (6) [P<0·01]	219·3±29·2 (6) [P=0·01]

of oxaloacetate did not significantly depress incorporation of ^{14}C from aspartic acid, but addition of oxaloacetate did diminish incorporation into protein of ^{14}C from alanine (Table 8; Fig. 2).

Fixation of carbon dioxide. We have found that when normal rat diaphragm is incubated in a $H^{14}CO_3^-$ buffer, ^{14}C is incorporated into protein (Table 9). Furthermore, this incorporation of ^{14}C into protein is stimulated by addition of pyruvate to the medium. When the gas phase is changed from $O_2 + CO_2$ to $N_2 + CO_2$ incorporation of ^{14}C from $NaH^{14}CO_3$ into protein is almost completely inhibited.

DISCUSSION

Effect of size of amino acid pool on rate of incorporation of ^{14}C from [^{14}C]amino acids

Incorporation of ^{14}C from both [^{14}C]-glutamic and -aspartic acid is much less than from other amino acids. Since free glutamic acid has been found in muscle in large quantities, a possible explanation of the poor incorporation of ^{14}C from glutamic acid is that the dilution of specific activity of [^{14}C]amino acids which takes place when they enter the amino acid pool in the muscle cell is greater for glutamic acid than for other amino acids. In addition, a portion of the [^{14}C]-glutamic and -aspartic acid may be converted into glutamine and asparagine before incorporation into protein. The specific activity of [^{14}C]-glutamine and -asparagine would therefore be less than that of added [^{14}C]-glutamic and -aspartic acid as a result of two dilutions. However, semi-quantitative experiments by us in which the free amino acids of fresh rat diaphragm have been separated by paper chromatography indicate that both glycine and alanine, as well as glutamic acid, are present in amounts considerably greater than those of other amino acids, and that

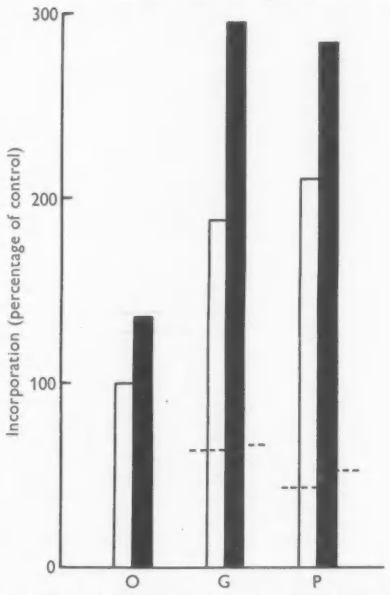

Fig. 1. Effect of addition of insulin, [^{14}C]glucose and [^{14}C]pyruvate to a medium containing [^{14}C]alanine on the incorporation *in vitro* of ^{14}C into protein of normal rat diaphragm. Figures on the ordinate express incorporation as a percentage of that found in the absence of insulin, glucose and pyruvate. Open blocks represent incorporation in the absence of insulin, solid blocks in the presence of insulin. O signifies that no oxidizable substrate had been added to the medium, G that [^{14}C]glucose was present and P that [^{14}C]pyruvate was present. Broken lines indicate the results obtained by Manchester & Young (1958) with unlabelled glucose and pyruvate.

Table 6. *Effect of the addition of α-oxoglutarate to the medium on the incorporation* in vitro *of ^{14}C from amino acids into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added oxoglutarate. The value of *P* for a difference which is significant is given in brackets. Number of observations is given in parentheses.

	Radioactivity in diaphragm protein	
[^{14}C]Amino acid added to medium	Control experiments (counts/min./disk)	Percentage of control value in the presence of added oxoglutarate
L-Alanine	691	90·7±4·2 (6)
Glycine	448	80·5±5·4 (6) [$P<0·02$]
L-Leucine	967	92·7±2·5 (6) [$P<0·05$]
L-Glutamic acid	105	50·3±3·3 (6) [$P<0·001$]
L-Aspartic acid	114	123·8±10·3 (6)

Table 7. *Effect of the addition of [^{14}C]α-oxoglutarate to a medium containing [^{14}C]glutamic acid on the incorporation* in vitro *of ^{14}C into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added [^{14}C]oxoglutarate. The value of *P* for a difference which is significant is given in brackets. Number of observations is given in parentheses.

Radioactivity in diaphragm protein	
Control experiments (counts/min./disk)	Percentage of control value in the presence of [^{14}C]α-oxoglutarate
28·5	403±16 (6) [$P<0·001$]

there appears to be little aspartic acid. Approximate figures (mg./100 g.) obtained by Tallan, Moore & Stein (1954) for the free amino acids of cat muscle are 30 for glutamic acid, 20 for alanine and for glycine, 5 for aspartic acid, 5 or below for other amino acids. Since incorporation of ^{14}C from both alanine and glycine is several times that from glutamic acid and aspartic acid, despite the relatively large pool of free alanine and glycine in the cell, reduction of specific activity is probably not alone responsible for the smallness of incorporation of ^{14}C from glutamic and aspartic acids when these substances enter the cell, although it almost certainly contributes to it. Probably a more important cause is that even in the absence of added substrate a portion of the [^{14}C]glutamic acid or [^{14}C]aspartic acid is transformed into a compound or compounds which cannot be incorporated into protein. It is perhaps not surprising that the two acids which give rise to least incorporation are the two most active in transamination (Braunstein, 1947).

Effect of transamination reactions on incorporation of ^{14}C from [^{14}C]amino acids

Braunstein & Kritzmann (1937) first showed that interconversion of amino and keto acids could take place by a process of transamination without the intermediate formation of ammonia. They studied three transaminase systems, namely glutamic acid–aspartic acid, glutamic acid–alanine and aspartic acid–alanine. Though subsequent work has shown that nearly all amino acids can take part in transamination reactions, the glutamic acid, aspartic acid and alanine systems are still considered as being the most active. For example, Awapara & Seale (1952) found that the formation of glutamic acid from oxoglutarate in a homogenate of rat-skeletal muscle, when aspartic acid or alanine was added, was over ten times that in the presence of leucine, isoleucine or valine. However, the glutamic, aspartic and alanine systems are believed not to be of similar activity. Braunstein (1947) states that in animal tissues the glutamic acid–aspartic acid transaminase enzyme is more active than the glutamic acid–alanine enzyme,

Table 8. *Effect of the addition of oxaloacetate to the medium on the incorporation* in vitro *of ^{14}C from amino acids into protein of normal rat diaphragm*

Results are expressed as a percentage of the control value found in the absence of added oxaloacetate. The value of P for a difference which is significant is given in brackets. Number of observations is given in parentheses.

[^{14}C]Amino acid added to medium	Radioactivity in diaphragm protein: Control experiments (counts/min./disk)	Radioactivity in diaphragm protein: Percentage of control value in the presence of added oxaloacetate
L-Alanine	593	59·2±2·8 (6) [$P<0·001$]
Glycine	638	89·5±4·1 (6) [$P=0·05$]
L-Leucine	1220	88·2±3·0 (6) [$P<0·02$]
L-Glutamic acid	157	40·7±1·9 (6) [$P<0·001$]
L-Aspartic acid	107	105·5±4·1 (12)

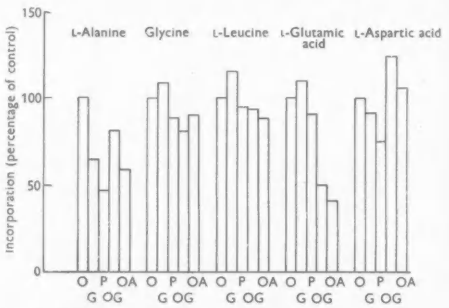

Fig. 2. Effect of addition to the medium of glucose, pyruvate, α-oxoglutarate and oxaloacetate on the incorporation *in vitro* of ^{14}C from [^{14}C]amino acids into protein of normal rat diaphragm. Figures on the ordinate express incorporation as a percentage of that found in the absence of added substrates. O signifies that no oxidizable substrate had been added to the medium, G, P, OG and OA that glucose, pyruvate, oxoglutarate or oxaloacetate respectively was present. Figures for the effect of glucose and pyruvate are taken from Manchester & Young (1958).

Table 9. *Effect of anaerobiosis and of addition of pyruvate on the incorporation* in vitro *of ^{14}C from $NaH^{14}CO_3$ into protein of normal rat diaphragm*

Number of observations is given in parentheses. The value of P is given where a difference between means is significant.

	Radioactivity in diaphragm protein (counts/min./disk)	
(1) No addition to medium		
(*a*) Gas phase O_2+CO_2 (95:5)	53·4±5·1 (3)	$P<0·001$
(*b*) Gas phase N_2+CO_2 (95:5)	4·0±1·1 (3)	
(2) Gas phase O_2+CO_2 (95:5)		
(*a*) No addition to medium	42·9±1·6 (6)	$P<0·02$
(*b*) Addition of pyruvate	49·5±1·7 (5)	

which in turn is more active than the aspartic acid–alanine system. This conclusion was confirmed by Awapara & Seale (1952), with a heart-muscle homogenate.

Transamination between [^{14}C]amino acids and unlabelled keto acids could lead to a diminution in incorporation of ^{14}C into protein by reducing the quantity of [^{14}C]amino acid available for protein synthesis. Moreover formation of unlabelled amino acid from added keto acid by transamination would depress incorporation of ^{14}C from [^{14}C]amino acid of the same kind. Other considerations apart, the more active the transaminating system and the more keto acid available, the smaller is the incorporation of ^{14}C from [^{14}C]amino acid to be expected.

Nisonoff, Barnes, Enns & Schuching (1954) showed that transamination could take place between an amino acid and the corresponding keto acid. The occurrence of such a process in diaphragm between [^{14}C]alanine and unlabelled pyruvate, as well as the formation of unlabelled alanine from pyruvate and other amino acids, would explain the diminution of incorporation of ^{14}C from alanine into protein which has been observed when pyruvate is added to the medium (Sinex *et al.* 1952; Manchester & Young, 1958). Addition of glucose instead of pyruvate also diminished incorporation from alanine, presumably because of the breakdown of glucose to pyruvate within the cell. Substances which inhibit transamination might therefore lessen the diminution of incorporation of ^{14}C from alanine which occurs when glucose or pyruvate is added to the medium.

The observation that addition of carbutamide or tolbutamide, which Bornstein (1957) found to inhibit a liver-transaminase system, fails to stimulate incorporation *in vitro* of ^{14}C from alanine in the presence of glucose or pyruvate might be taken as some evidence against the idea that the diminution of incorporation of ^{14}C in the presence of glucose and pyruvate is due to a dilution of [^{14}C]alanine by unlabelled alanine formed from pyruvate by transamination. On the other hand, it is possible that neither carbutamide nor tolbutamide inhibits muscle transaminase, as opposed to liver transaminase, or that these substances have other actions which result in an inhibition of incorporation. Incorporation of ^{14}C from [^{14}C]amino acid is greatly depressed by many well-known metabolic inhibitors (K. L. Manchester & F. G. Young, unpublished work), and it is possible that carbutamide and tolbutamide inhibit the process of incorporation at concentrations lower than those at which they can exert any putative effect on transaminase-enzyme systems.

Effect of [^{14}C]*-glucose and -pyruvate.* The observation that ^{14}C from [^{14}C]-glucose and -pyruvate is incorporated into protein under the conditions of our experiments (Fig. 1) agrees with the idea that transamination takes place in rat diaphragm. The only [^{14}C]amino acids likely to be formed from [1-^{14}C]pyruvate are alanine and, possibly, serine and glycine, since any amino acids formed by reactions involving preliminary decarboxylation of pyruvate would be unlabelled. Thus aspartic acid or glutamic acid formed from [1-^{14}C]pyruvate by operation of the tricarboxylic acid cycle would be unlabelled unless the $^{14}CO_2$ liberated in the cycle was subsequently fixed into oxaloacetate. No such limitations would apply, however, to [U-^{14}C]-glucose, and it is possible that [^{14}C]alanine, glutamic acid and aspartic acid are formed from [^{14}C]glucose, unless, as is argued below, the appropriate keto acids formed from glucose, that is oxoglutarate and oxaloacetate, are generated in a locus which renders them not readily available to transaminase enzymes.

Effect of oxoglutarate. Manchester & Young (1958) found that although addition of glucose and pyruvate depressed incorporation into protein of ^{14}C from alanine, these substrates did not depress incorporation of ^{14}C from glutamic acid, an occurrence which might have been expected if transamination was taking place. They considered the possibility that because the enzymes of the tricarboxylic acid cycle are situated within the mitochondrion, oxoglutarate formed by oxidation of glucose or pyruvate might not be available for transamination by enzymes outside the mitochondrion. Free keto acids are present in tissues in only small quantities, and it is perhaps significant that Cavallini & Frontali (1954) found in rat muscle three times as much pyruvate as oxoglutarate (0·3 and 0·1 mg./100 g. respectively).

Addition of unlabelled oxoglutarate depresses incorporation of ^{14}C from glutamic acid (Fig. 2). Clearly, under these conditions [^{14}C]glutamic acid is diluted in the cell either by transamination with unlabelled oxoglutarate to form unlabelled glutamic acid, or by formation of glutamic acid from added oxoglutarate and, say, alanine from the cell pool, or by a combination of both processes. That unlabelled glutamic acid is being formed from added oxoglutarate by transamination is suggested by the observation that incorporation of ^{14}C from glycine and leucine is slightly depressed when oxoglutarate is added.

Incorporation of ^{14}C from [5-^{14}C]oxoglutarate could result from two possible processes. One of these is the formation of [^{14}C]glutamic acid by transamination or by the glutamic dehydrogenase reaction (Dewan, 1938). The second possible process is the production of [^{14}C]aspartic acid from [^{14}C]oxaloacetate which had itself been formed by the oxidation of [^{14}C]oxoglutarate in the tricarboxylic acid cycle.

In view of these possibilities, it is surprising that addition of oxoglutarate does not depress incorporation of ^{14}C from either aspartic acid or alanine under the conditions of our experiments. For this fact we have at present no adequate explanation.

Effect of oxaloacetate. The addition of oxaloacetate depresses incorporation into protein of ^{14}C from glutamic acid and, to a much less extent, from glycine and leucine (Fig. 2), presumably by a process of transamination in which aspartic acid and [^{14}C]keto acids are formed. Since the [^{14}C]keto acids cannot be incorporated into protein as such, incorporation of ^{14}C is depressed.

Addition of oxaloacetate, unlike addition of oxoglutarate, depresses incorporation of ^{14}C from alanine (Fig. 2), though whether this is due to the addition of oxaloacetate itself or to the formation of pyruvate from oxaloacetate by decarboxylation inside the cell, is not certain. As with oxoglutarate, however, addition of oxaloacetate does not depress incorporation of ^{14}C from aspartic acid (Fig. 2), and again no satisfactory explanation can at present be offered.

The behaviour of aspartic acid is peculiar in several ways. Apart from alanine, it is the only amino acid from which incorporation of ^{14}C is depressed by addition of pyruvate, and yet incorporation of ^{14}C from aspartic acid is not diminished in the presence of oxoglutarate or oxaloacetate. Incorporation of ^{14}C from aspartic acid is extremely low despite the fact that the cell pool appears to be small, and the [^{14}C]acid may therefore suffer only a relatively small dilution on entering the cell.

Incorporation of $^{14}CO_2$

Fixation of carbon dioxide by pyruvate to form oxaloacetate in animal tissues was first demonstrated by Krebs & Eggleston (1940) in minced pigeon liver. Ochoa (1945), using an extract of washed acetone-dried pig heart, showed that the enzymic decarboxylation of oxalosuccinate to oxoglutarate and carbon dioxide was reversible. Stern (1948), using a wide variety of animal tissues, was able to demonstrate fixation of carbon dioxide into oxaloacetate in only pigeon liver and, to a smaller extent, sheep-kidney cortex, though many tissues formed citric acid from oxoglutarate. In our experiments the entry of ^{14}C from $NaH^{14}CO_3$ into diaphragm protein is strong evidence that fixation of carbon dioxide can take place in mammalian muscle. Moreover, incorporation of ^{14}C into protein indicates that fixation into oxaloacetate is almost certainly involved with the subsequent formation of [^{14}C]aspartic acid. Fixation of carbon dioxide by oxoglutarate or oxalosuccinate would not result in the formation of [^{14}C]glutamic acid or [^{14}C]aspartic acid, and would therefore not provide for the formation of [^{14}C]protein.

SUMMARY

1. Incorporation into rat-diaphragm protein *in vitro* of ^{14}C from a medium containing [^{14}C]alanine is greater when [^{14}C]-glucose or -pyruvate is present in the medium than in their absence. Addition of insulin further increases incorporation of ^{14}C in both the presence and the absence of [^{14}C]-glucose or -pyruvate.

2. Addition of oxoglutarate to the medium substantially depresses incorporation into protein of ^{14}C from [^{14}C]glutamic acid, and slightly depresses that from alanine, glycine and leucine. Incorporation of ^{14}C from aspartic acid is somewhat enhanced under these conditions.

3. Incorporation into diaphragm protein of ^{14}C from a medium containing [^{14}C]glutamic acid is vastly increased when [^{14}C]oxoglutarate is also added.

4. Addition of oxaloacetate depresses severely incorporation into protein of ^{14}C from [^{14}C]-alanine and -glutamic acid, and slightly from glycine and leucine. Incorporation of ^{14}C from aspartic acid is not affected.

5. In the presence of $NaH^{14}CO_3$, ^{14}C is incorporated into protein of normal rat diaphragm.

6. Incorporation into protein of normal rat diaphragm of ^{14}C from [^{14}C]alanine is depressed in the presence of carbutamide or tolbutamide whether or not glucose or pyruvate is present.

7. The results are largely, but not entirely, explicable on the assumption that transamination reactions, together with carboxylation, can proceed in isolated rat diaphragm under the conditions of our experiments.

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The Labelling by [^{14}C]Amino Acids of Cell-Sap Protein in a Cell-Free System from Guinea-pig Liver

THE SITE OF ORIGIN OF LABELLED PROTEIN

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It is now established that amino acids are incorporated into the protein of the microsome fraction of tissues such as liver at a more rapid rate than into the protein of other subcellular fractions both in the intact animal (e.g. Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Hultin, 1950; Keller, 1951) and in cell-free systems (e.g. Siekevitz, 1952; Zamecnik & Keller, 1954). These findings suggest that the microsome fraction is of importance as a site of synthesis of other cytoplasmic proteins. While no evidence has been obtained showing that the microsome fraction is the site of origin of all cytoplasmic proteins, some evidence has been obtained which indicates that it is the site of synthesis of certain specific soluble proteins (e.g. Peters, 1957; Rabinovitz & Olson, 1956). It would be therefore of great value if the synthesis of specific soluble proteins could be studied in entirely cell-free systems. However, few instances have been reported of an unequivocal demonstration of either the labelling or net synthesis of a specific soluble protein in a cell-free system. In addition, a previous study made in this Laboratory (Simkin & Work, 1957*b*) has suggested that there might be some difference between the incorporation of amino acids into the protein of the microsome fraction of intact cells of guinea-pig liver and the incorporation into the microsome protein of a corresponding cell-free system. A microsome–cell-sap system of the type described by Zamecnik & Keller (1954) was used for these studies, and we reported that some soluble proteinaceous material present in the cell-sap fraction became labelled to a significant extent upon incubation. This finding prompted an investigation into the origin of the soluble protein which becomes labelled in this system. The present paper provides evidence suggesting that the microsome fraction does act as site of origin of the labelled soluble protein, and data are reported relating to factors affecting the passage of radioactivity from microsome to cell-sap fractions. This study forms part of a more general investigation into the significance of amino acid incorporation in cell-free systems, in the course of which it is hoped to discover whether specific soluble proteins are formed in such systems and whether the microsome fraction is the source of origin of such proteins.

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EXPERIMENTAL

Animals. As in earlier studies (Simkin & Work, 1957*b*), young guinea pigs (400–500 g.), which had been starved overnight, were used.

Materials. Reagents were prepared as described by Simkin & Work (1957*b*), except that in some experiments a purified preparation of [^{14}C]chlorella-protein hydrolysate was used (see below). Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) were purchased from the Sigma Chemical Co.

Purification of chlorella-protein hydrolysates. Hydrolysates of [^{14}C]chlorella protein were prepared as previously described. The amino acids were adsorbed on a column of Zeo-Karb 225 (H^+) and eluted with aq. 0·3 M-NH_3. The NH_3 was removed *in vacuo* and the residue dissolved in water. The use of such purified preparations resulted in slightly lower protein specific-activity values being obtained compared with values with chlorella-protein hydrolysates not subjected to this treatment. In other respects the results with both kinds of preparation were very

similar in studies both of incorporation into protein and of transfer of radioactivity from labelled microsome material. Zero-time values for cell-sap protein in incorporation studies were, however, lower when purified preparations were used. We have thus been unable to confirm, under the conditions employed in the present study, the finding of Craddock & Dalgliesh (1957) that purification of [^{14}C]-chlorella-protein hydrolysates is attended by a large reduction in the incorporation of radioactivity into protein.

Preparation of ^{14}C-labelled microsome fractions. Microsome–cell-sap preparations were prepared from 0·25 M-sucrose homogenates of liver as described by Simkin & Work (1957*b*). For the preparation of labelled microsome material, the microsome–cell-sap preparations were mixed with the same incubation medium as used earlier (for details, see Fig. 1), except that in some instances the concentration of [^{14}C]chlorella-protein hydrolysate was one-half of that used previously, although the total radioactivity added was the same as before. From data reported earlier (Simkin & Work, 1957*b*), it may be calculated that the amount of labelled amino acid added is only about 2·5% of the unlabelled amino acid already present in the system, and a reduction of the quantity of amino acid added should therefore make no appreciable difference to the total concentration. The conditions of incubation were those used previously. The total volume of the cell-free systems used was in general 14·4 ml. as before, but in some cases, where the labelled microsome material was not subsequently reincubated, the total volume was reduced to 3·6 ml. (in such cases, sucrose–casein hydrolysate was added as described below before centrifuging). After incubation, the mixtures were rapidly cooled in ice, and microsome and cell-sap fractions were separated by centrifuging without delay for 45 min. at 40 000 rev./min. ($g_{av.}$ 105 000) in Rotor 40 of a Spinco model L ultracentrifuge.

Preparation of cell-sap media for reincubation of microsomes. When cell-sap preparations were required for the reincubation of labelled microsome material, the microsome–cell-sap preparations (10 ml.) were mixed with the usual incubation medium (4·4 ml.), from which, as indicated in the Results section, [^{14}C]amino acids, 3-phosphoglycerate or $MgCl_2$ were omitted as required. Cell-sap fractions were obtained by centrifuging for 45 min. as described above, without prior incubation.

Reincubation of labelled microsome fractions. The surface of the labelled microsome pellet, prepared as described above, was washed twice with 2–3 ml. of 0·25 M-sucrose. The microsome material was then resuspended in a volume of cell sap (see above) equal to that from which it had been separated. Resuspension was effected in a Potter–Elvehjem-type homogenizer with a Teflon (polytetrafluoroethylene) pestle (Simkin & Work, 1957*a*) which was driven at about 1000 rev./min. Samples (3·5 ml.) of the suspensions were then incubated exactly as described earlier. Approximately 100 min. elapsed between the end of the first incubation (preparation of labelled microsomes) and the start of the second incubation (with fresh cell sap); during this time, all operations were carried out at 0–5°.

Separation of microsome and cell-sap fractions and isolation of protein for radioactivity measurement. At the end of incubation, approximately 2·5 vol. of an ice-cold 0·25 M-sucrose solution containing unlabelled amino acids (10 mM with respect to casein hydrolysate N) was added to the incubation mixtures. The diluted incubation mixtures were cooled in ice and then centrifuged without delay for 45 min. at 40 000 rev./min. The supernatants were removed as described earlier (Simkin & Work, 1957*a*) and taken as the cell-sap fractions. The surface of the microsome pellets was washed with 0·25 M-sucrose as described previously. Both microsome and cell-sap samples were frozen solid and stored in this state (usually at −35°) until taken for isolation of protein.

Microsome pellets were resuspended in iced water (5 ml.) by homogenization before precipitation of protein. Protein was precipitated with trichloroacetic acid (TCA), reprecipitated, purified and plated at infinite thickness as described by Simkin & Work (1957*a*).

Preparation of the trichloroacetic acid-soluble fraction of the cell sap for radioactivity measurement. The TCA-soluble fraction of the cell sap was prepared and diluted (tenfold) as described by Simkin & Work (1957*b*). A sample (0·5 ml.) was added to a 4·7 cm.^{2} nickel planchet which contained a few drops of diluted detergent and a disk of lens paper, and water was then removed *in vacuo*.

Determination of radioactivity. Samples were counted to an accuracy of at least ±5% with an end-window Geiger counter. Under the conditions used, 1 cm.^{2} of methacrylate resin of 10^{-3} μc/mg. gives 1000 counts/min. at infinite thickness.

RESULTS

Incorporation of [^{14}C]amino acids into microsome and cell-sap protein in a cell-free system

The time course of incorporation of a mixture of [^{14}C]amino acids into the protein of both microsome and cell-sap fractions in a microsome–cell-sap system from guinea-pig liver has been described (Simkin & Work, 1957*b*) (see also Fig. 1). There is an approximately linear increase in the specific activity of the microsome protein for about 20 min. The specific activity of the cell-sap protein increases only slightly during the first 10 min. of incubation, but then shows a rapid increase during the succeeding 10 min. period. After incorporation into microsome protein has apparently ceased, there is a further period during which the protein of the cell sap continues to increase in radioactivity. While the length of time during which amino acids are incorporated into microsome protein varies from preparation to preparation (20 ± 5 min.), the specific-activity curve for cell-sap protein always follows the specific-activity curve for microsome protein in an essentially similar manner to that shown in Fig. 1.

Because the amount of protein present in the cell-sap fraction is greater than that in the microsome fraction in this system (Simkin & Work, 1957*b*), it is preferable to plot data in terms of total amino acid uptake rather than as specific activity. The ratio of cell-sap protein to microsome protein is 3·8:1 ± S.D. 0·4 (16 preparations). The reason why this ratio is higher than that found when a more refined method of cell fractionation

(de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) is applied to a guinea-pig-liver homogenate is that losses of microsome material occur during the preparation of the microsome–cell-sap fractions. A comparison of the total number of counts in the protein of microsome and cell-sap fractions can be made by multiplying the specific-activity values of the cell-sap protein by the ratio of cell-sap protein to microsome protein; in the experiment shown in Fig. 1, this ratio was 4:1. When the results of a typical experiment are plotted in this manner (Fig. 1), it is seen that after 20 min. incubation the radioactivity of the cell-sap protein forms a substantial proportion (about 30 % in this case) of the radioactivity present in the protein of the system as a whole. Preliminary results indicate that a similar distribution of protein radioactivity is obtained with microsome–cell-sap preparations from rat liver.

Radioactivity of cell-sap protein after reincubation of labelled microsome fractions with unlabelled cell sap

In experiments such as that illustrated by Fig. 1, it is impossible to determine whether the radioactive protein appearing in the cell sap is derived from the microsome fraction or whether it is synthesized by a microsome-independent process. Further experiments were designed to clarify this point.

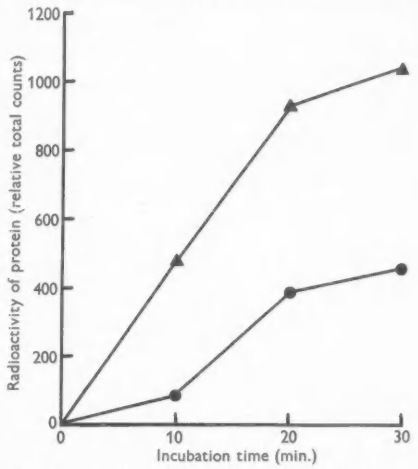

Fig. 1. Incorporation of [^{14}C]amino acids into microsome (▲) and cell-sap (●) protein upon incubation of a cell-free system prepared from guinea-pig liver. The incubation mixture contained: microsome + cell-sap fractions; KCl (20 mM); $KHCO_3$ (25 mM); potassium phosphate buffer, pH 7·8 (15 mM); $MgCl_2$ (2·5 mM); potassium 3-phosphoglycerate (10 mM); [^{14}C]chlorella-protein hydrolysate (0·09–0·18 mM, 0·8 μc/ml.). Incubation was carried out at 37° in an atmosphere of $N_2 + CO_2$ (95:5, v/v). For other details see text. The microsome values are plotted in terms of counts/min./cm.^{2} and the cell-sap values as 4 × counts/min./cm.^{2} (see text). The values have been corrected for zero-time uptake.

If cell sap was isolated from a microsome–cell-sap preparation, and then supplemented with [^{14}C]-amino acids, cofactors, etc., in the usual way and incubated, there was no significant incorporation of amino acid into the cell-sap protein. Alternatively, if a microsome–cell-sap preparation was incubated for 10 or 20 min. with [^{14}C]amino acids in the usual way and then centrifuged to remove microsomes, the cell sap thus obtained showed no further increase in the specific activity of its protein upon subsequent incubation. It thus seemed probable that the microsome material played an essential part in the formation of cell-sap protein in this cell-free system.

A direct test of this hypothesis could be made by incubation of [^{14}C]labelled microsome material with fresh cell sap. The protein-specific-activity curves of Fig. 1 show that after 10 min. incubation microsome protein is labelled to a substantial extent, whereas the cell-sap proteins contain little ^{14}C. It seemed likely therefore that microsome material isolated after 10 min. incubation in the presence of [^{14}C]amino acids would be particularly suitable for the study of transfer of radioactivity from microsomes to cell-sap protein, and such microsome material has been used throughout unless otherwise stated.

The basic plan of the experiments to be described was as follows. A microsome–cell-sap preparation was incubated with [^{14}C]amino acids in the usual way for 10 min. The incubation mixture was centrifuged, and the labelled microsomes so obtained were resuspended in unlabelled non-incubated cell sap. The latter was always prepared, as described in the Experimental section, from the same microsome–cell-sap preparation as that used to obtain the labelled microsomes, and in all cases contained the customary buffers and potassium chloride. When the cell sap used for resuspension was supplemented with 3-phosphoglycerate and Mg^{2+} ions, there was an increase in the specific activity of the cell-sap protein upon incubation under the customary conditions (Fig. 2). This was accompanied by a decrease in the specific activity of the microsome protein (changes in microsome-protein activity are considered more fully in a later section). It will be seen that the cell-sap protein increases in activity over a period of about 20 min., and thereafter little or no increase is found. With some preparations the increase in activity was approximately linear with time, but more often the increase during the first 10 min. of incubation was greater than that occurring during the succeeding 10 min. period.

If 3-phosphoglycerate, which serves as substrate for an ATP-generating system, was omitted from the cell-sap medium used for resuspension of labelled microsomes, the increase in the specific activity of the cell-sap protein was much smaller than in the corresponding control (Fig. 3). The effect of omission was less marked during the first 10 min. of incubation than during the succeeding 10 min. period. The specific-activity values for cell-sap protein isolated after 20 min. incubation in the absence of 3-phosphoglycerate were only 16, 23 and 24 % of those found in its presence. Similarly, if Mg^{2+} ions were omitted from the incubation medium, the specific activity of cell-sap protein was lower than in controls (Fig. 4). The effect of omission of Mg^{2+} ions, though again less marked during the first 10 min. of incubation, was not as great as that resulting from the absence of 3-phosphoglycerate: specific-activity values for cell-sap protein after incubation for 20 min. without Mg^{2+} ions were 42, 51 and 58 % of the controls with Mg^{2+}. In several experiments, labelled microsomes were incubated for 15 or 20 min. with fresh cell sap but without either 3-phosphoglycerate or Mg^{2+}. The cell sap was then separated (without dilution with sucrose–casein hydrolysate) and reincubated at once (in the absence of microsome material) with the customary concentration of either 3-phosphoglycerate or Mg^{2+} ions respectively. In neither case was there an increase in the specific activity of the cell-sap protein as a result of the final incubation. The results of a typical experiment of this type are shown in Fig. 5.

In several experiments microsome material was isolated after incubation with [^{14}C]amino acids for 20 min. instead of the customary 10 min. When the labelled microsomes were reincubated with fresh cell-sap media similar to those described above, it

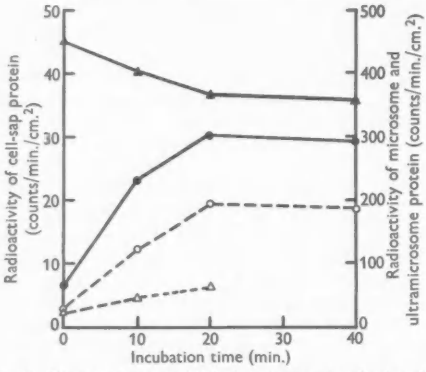

Fig. 2. Microsome material isolated after incubation for 10 min. with [^{14}C]amino acids, as described for Fig. 1, was reincubated with an unlabelled non-incubated cell-sap preparation (see text). The final concentrations of various additions and conditions of incubation were as for Fig. 1. After incubation, the cell-sap (●) and microsome (▲) fractions were separated and their protein isolated. Samples of the cell-sap fractions were also centrifuged at 105 000 $g_{av.}$ for 6 hr. before precipitation of protein to obtain recentrifuged cell-sap (○) and ultramicrosome (△) fractions. The ultramicrosome-protein value plotted at 20 min. is for material isolated from the pooled 20 and 40 min. samples

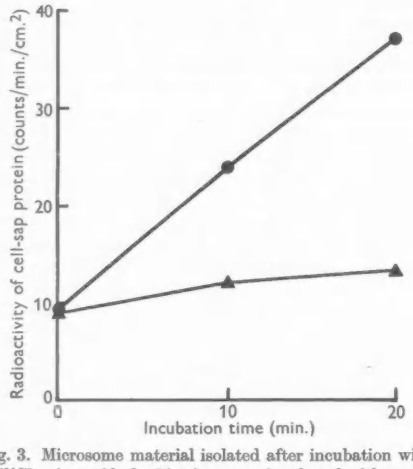

Fig. 3. Microsome material isolated after incubation with [^{14}C]amino acids for 10 min. was reincubated with a cell-sap preparation with (●) or without (▲) the addition of 3-phosphoglycerate. For details see Fig. 2.

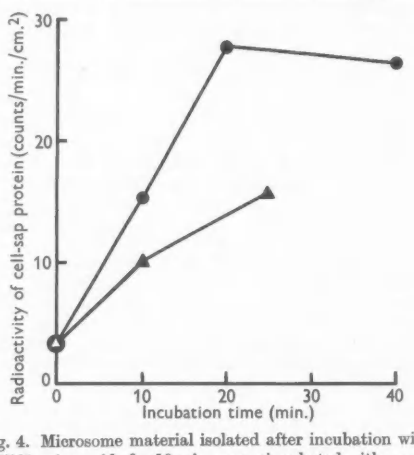

Fig. 4. Microsome material isolated after incubation with [^{14}C]amino acids for 10 min. was reincubated with a cell-sap preparation with (●) or without (▲) the addition of Mg^{2+}. For details see Fig. 2.

was found that again there was an increase in the specific activity of the cell-sap protein. The increase, which occurred mainly during the first 10 min. of incubation, was somewhat smaller than that obtained under similar conditions with the same microsome material labelled initially for only 10 min. When 3-phosphoglycerate was omitted from the reincubation medium, the specific activity of the cell-sap protein was again lower than in the control, being about 50 % of the control value with 20 min. periods of incubation. The effect of omission of 3-phosphoglycerate was thus less marked than with microsome material initially labelled for 10 min.

If samples of cell sap, obtained by centrifuging incubated or non-incubated mixtures of labelled microsomes and fresh cell sap, were dialysed extensively at 2° against 0·14 M-sodium chloride–0·02 M-tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer, pH 7·2, before precipitation of protein with TCA, there was no significant change in the specific activity of the protein isolated compared with undialysed controls. When similar samples of cell sap were recentrifuged at 105 000 $g_{av.}$ for 6 hr., there was some reduction in the specific activity of the protein isolated from the resulting supernatant compared with protein from the original material (Fig. 2), but at least 50 % of the total protein radioactivity remained in the supernatant, except for the zero-time sample when the proportion was somewhat less. Centrifuging in such a manner would be expected to sediment completely particles possessing the reported characteristics (see, for example, Petermann & Hamilton, 1957) of liver cytoplasmic-ribonucleoprotein particles. The brown-coloured pellet which sedimented under these conditions contained about 20 % of the protein present in the original cell sap. The specific activity of this ultramicrosomal protein, while somewhat higher than that of the protein not sedimenting, was much lower than that of the corresponding microsome protein (Fig. 2).

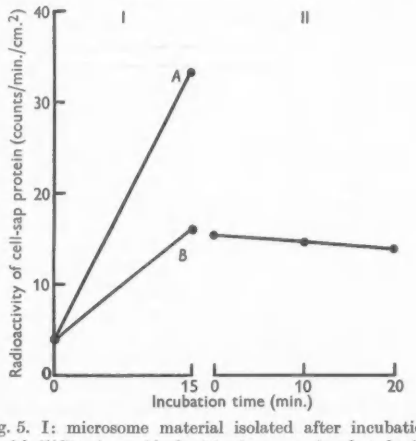

Fig. 5. I: microsome material isolated after incubation with [^{14}C]amino acids for 10 min. was reincubated with a cell-sap preparation with (*A*) or without (*B*) the addition of Mg^{2+} ions (for details see Fig. 2). II: cell sap which had been isolated from a separate sample of *B* after incubation was incubated (in the absence of microsomes) after the addition of Mg^{2+} ions to the usual final concentration.

Changes in the radioactivity of the protein of labelled microsomes upon reincubation

When labelled microsome material was reincubated with the customary cell-sap medium containing both 3-phosphoglycerate and Mg^{2+} ions, there was a decrease in the specific activity of the microsome protein. As a result of 20 min. incubation the mean fall in specific activity amounted to 22 % ± S.D. 4·0 (11 values) of the initial value. Because of the relatively small decrease in activity, it is difficult to determine with accuracy either the extent of the fall in activity or the nature of the change with time. However, in general the specific activity of the microsome protein decreased gradually for as long as the activity of the cell-sap protein increased, and then remained approximately constant (Fig. 2). The mean ratio, in terms of specific activity, of microsome protein decrease to cell-sap protein increase was 3·9:1 ± S.D. 0·9 (11 values, 20 min. incubation). If this ratio is calculated in terms of total counts rather than as specific activity as discussed earlier, using the mean value quoted above of 3·8:1 for the ratio of microsome protein to cell-sap protein, it will be apparent that there is an approximate equivalence between the decrease in microsome-protein radioactivity and increase in cell-sap-protein radioactivity. Because of the possible experimental errors, it cannot be established whether this equivalence is strictly held for each preparation.

There was some variation from preparation to preparation in the effects upon the specific activity of microsome protein of omission of 3-phosphoglycerate or Mg^{2+} from the media used to reincubate labelled microsomes. Thus, when 3-phosphoglycerate was absent, the decrease in specific activity was only 25–30 % of that found in the corresponding control in two preparations, but was similar to the value for the control in a third preparation. The percentage decrease in activity in the absence of Mg^{2+} was similar to that found for the corresponding control in two preparations, but in a third case the decrease was only about 50 % of that obtained in the control. If the small number of preparations studied and possible experimental errors are taken into consideration, no conclusions

can be drawn concerning the changes in the specific activity of microsome protein under these conditions.

The protein of microsome material incubated initially with [^{14}C]amino acids for 20 min. also decreased in specific activity upon reincubation with fresh cell sap. The percentage decrease was similar to that found with material labelled initially for 10 min. As the specific activity of the cell-sap protein was not very different in the two cases, the fall in microsome-protein radioactivity was thus larger than could be accounted for by the increase in that of cell-sap protein. With the one preparation studied, omission of 3-phosphoglycerate from the reincubation medium reduced the magnitude of the decrease in microsome-protein activity.

Factors other than experimental error might also play a part in producing variation in the level of microsome-protein radioactivity during reincubation. For example, radioactive protein could be degraded to TCA-soluble material, and there is in fact some evidence suggesting that this may occur. Thus, as previously reported (Simkin & Work, 1957*b*), there is a small increase of TCA-soluble amino N (as measured colorimetrically after reaction with β-naphthoquinonesulphonate) during incubation of the microsome–cell-sap system. Small changes in protein level may occur, but are not easy to detect. The fact that a small decrease in the specific activity of cell-sap protein has been found to occur after incorporation has ceased (see, for example, Figs. 2, 4 and 5) could be taken as evidence for protein degradation. The microsome–cell-sap fraction of liver is known also to contain some proteolytic enzymes (e.g. Rademaker & Soons, 1957).

Reincubation of labelled microsome fractions with fresh cell sap supplemented with [^{14}C]*amino acids*

A number of experiments were carried out to determine whether microsome material labelled with [^{14}C]amino acids for 10 min. in the usual way, and then isolated, was still capable of incorporating amino acids into its protein when resuspended in fresh cell sap and reincubated. These studies could also reveal whether it was possible for amino acids incorporated into microsome protein during reincubation to be transferred to the cell-sap protein. When labelled microsome material was resuspended in a fresh cell-sap medium supplemented with [^{14}C]amino acids, there was an increase in the specific activity of the protein of the microsome fraction in contradistinction to the fall observed in media without [^{14}C]amino acids. The increase in the specific activity of the cell-sap protein under these conditions was about twice that occurring in media not supplemented with [^{14}C]amino acids (Fig. 6). The additional increments in specific activity of both cell-sap and microsome fractions on reincubation in labelled media were, however, smaller (e.g. for cell sap, about one-half during the first 10 min. of incubation) than those obtained as a result of incubation of another sample of the original microsome–cell-sap system beyond the customary 10 min. period (Fig. 6). Systems containing microsome and cell-sap fractions separated and recombined without a preliminary incubation step have been found capable of incorporating amino acids into both microsome and cell-sap proteins at rates essentially similar to the corresponding system which had not been subjected to separation and recombination. This suggests that the decreased incorporation into protein found when labelled microsome material is reincubated may result from some kind of damage occurring during the preliminary incubation step.

It appeared possible that mixtures of labelled microsome material and fresh unlabelled cell sap might contain appreciable quantities of free [^{14}C]-amino acids which would be introduced into the system as a result of adsorption, etc., on microsomes during the initial labelling step. Some estimate of the extent of this contamination was obtained by comparing the TCA-soluble radioactivity of cell sap separated from mixtures of labelled microsomes and unlabelled cell sap with that of cell sap separated from the system used to prepare labelled microsomes. Some TCA-soluble radioactivity was found in cell sap from mixtures

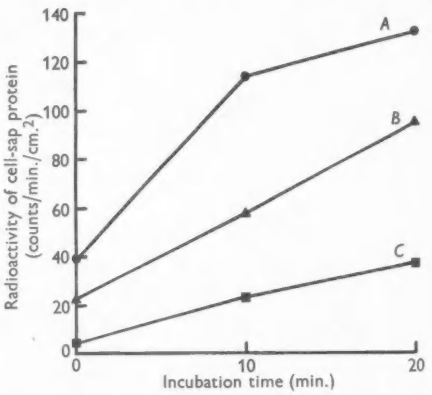

Fig. 6. A microsome–cell-sap preparation was incubated for 10 min. with [^{14}C]amino acids (see Fig. 1). One sample of the incubation mixture was then incubated further (*A*); zero time represents the beginning of the second period of incubation. Microsome material was isolated from a separate sample of the incubation mixture and reincubated with a fresh cell-sap preparation with (*B*) or without (*C*) the addition of [^{14}C]amino acids (for details see Fig. 2).

of labelled microsomes and unlabelled cell sap, but the amount present, though rather variable, was always small, being 2·7–6·6 % (mean 4·0 % ± S.D. 1·35, 10 experiments) of the quantity present in the original labelling system, and remained essentially constant during the customary period of incubation.

The effect of omission of either 3-phosphoglycerate or Mg^{2+} ions from reincubation media supplemented with [^{14}C]amino acids upon amino acid incorporation into protein has not been studied, but it has been found that omission of either from the original labelling system virtually abolishes incorporation.

Reincubation of labelled microsome fractions with sucrose-diluted cell-sap media

A study was made of the effect of replacing part of the cell-sap media used for the reincubation of labelled microsomes by 0·25 M-sucrose. Microsome material, labelled with [^{14}C]amino acids for 10 min. as before, was resuspended in a medium in which 75 % of the cell sap had been replaced by 0·25 M-sucrose; 3-phosphoglycerate, Mg^{2+}, etc. were maintained at the usual final concentration. This medium was always supplemented (for the reason given below) with (in terms of final concentration) ATP (mM), GTP (0·15 mM) and additional Mg^{2+} (total, 5 mM), but despite this the increase in the radioactivity of the cell-sap protein after 20 min. incubation was, in terms of total number of counts in protein, only about one-quarter of the increase found for the corresponding control (i.e. undiluted cell-sap medium without ATP, GTP and additional Mg^{2+}). There was only a very small decrease in the specific activity of the microsome protein upon incubation with such diluted media, the decreases found after 20 min. incubation being only 13 and 25 % of the decreases occurring in control media. In an experiment in which labelled microsomes were resuspended in a sucrose-diluted cell-sap medium supplemented with [^{14}C]amino acids (the amount of radioactivity added being one-quarter of normal in order to maintain the specific activity of the free amino acid pool approximately constant), it was found that amino acids were incorporated into the protein of both microsome and cell-sap fractions, but at rates much slower than those which were found with the control medium.

Investigations of amino acid uptake into microsome protein in systems in which non-incubated microsomes were resuspended in the sucrose–cell-sap medium had shown that, unless ATP and GTP were added, there was virtually no incorporation of amino acid into microsome protein (cf. Keller & Zamecnik, 1956). The addition of these two compounds and further Mg^{2+} ions (at concentrations given above) stimulated incorporation, but it has not proved possible so far to obtain a rate of incorporation similar to that of the control system (i.e. the customary labelling system). The restoration of incorporation into microsome protein brought about by these compounds has been somewhat variable: for 20 min. periods of incubation, values of 20, 37 and 63 % of control incorporation were obtained with three separate microsome–cell-sap preparations.

DISCUSSION

The data reported support the hypothesis that in a microsome–cell-sap system the protein of the microsome fraction acts as precursor of some protein present in the cell sap. Significant labelling of cell-sap protein is found only when microsome material is present. When labelled microsome material is reincubated with unlabelled cell-sap preparations, the time course of the increase in the specific activity of cell-sap protein (e.g. Fig. 2) is essentially that which would be expected from the characteristics of amino acid incorporation into the original microsome–cell-sap system (e.g. Fig. 1). The decreases in the radioactivity of microsome protein obtained in a variety of unlabelled media have been found in most cases to be approximately equivalent to the increases in the radioactivity of the cell-sap protein; in the remaining instances the decreases found have been larger and not smaller than those expected. It seems reasonable to assume that a large proportion of the TCA-precipitable radioactive material present in the cell sap of the systems studied is in the form of soluble protein, for dialysis before precipitation does not affect the specific activity of the material, and centrifuging under conditions chosen to remove particles of the size of ribonucleoprotein granules and ferritin fails to sediment a substantial proportion of the TCA-precipitable radioactivity.

Although there appears to be transfer of label from microsome to cell-sap protein, it has not yet been unequivocally established whether label is transferred in the form of polypeptide or in the form of a relatively low-molecular-weight intermediate which would subsequently be polymerized in the cell sap. Such evidence as is available tends to favour the transfer of a large molecule. Free amino acids are excluded as intermediates in the transfer of radioactivity because no significant incorporation into protein occurs when cell sap, which contains appreciable quantities of free amino acids (about 7 mM in incubation mixture, Simkin & Work, 1957*b*), is incubated alone. Further, the possibility that the intermediate can be a compound in rapid equilibrium with the free amino acid pool is also excluded, because any such compound would be diluted very greatly if

released into unlabelled cell sap. Korner & Tarver (1957) have shown that microsome protein labelled *in vivo* can release under certain conditions small quantities of amino acid in a form that is not in rapid equilibrium with the free amino acid pool. Experiments already described (see, for example, Fig. 5) which were designed to test whether it is possible to show accumulation of such intermediates and their participation in the labelling of cell-sap protein gave negative results. Such experiments are not, however, conclusive because the release of intermediates from the microsome fraction might require the presence of 3-phosphoglycerate or Mg^{2+} ions; alternatively, the material released might not accumulate in the system.

The process resulting in the transfer of radioactivity from microsome to cell-sap protein appears to be an enzymic one, for omission of 3-phosphoglycerate, which would interfere with the generation of ATP, or of Mg^{2+} ions, markedly affects the process. The labelling of cell-sap protein which still takes place in media to which these compounds had not been added may, at least in part, be due to the presence of small quantities of the compounds which had been introduced by the labelled microsome material (cf. contamination with TCA-soluble radioactivity). The finding that substitution of 75% of the cell sap by 0·25M-sucrose reduces markedly the labelling of cell-sap protein may indicate that cell-sap enzymes are concerned with the process of passage of radioactivity from microsome to cell-sap protein; the reduction in labelling found might be due to interference with the generation of ATP, or perhaps to reduction in the concentration of enzymes or cofactors mediating in the transfer process. In one experiment it was found that replacement of the sucrose plus ATP and GTP used for dilution of cell sap by a dialysate of cell sap (of concentration equivalent to that normally present) did not result in an appreciable increase in the labelling of cell-sap protein. It is known, however, that incorporation of amino acid into protein is almost abolished by omission of either 3-phosphoglycerate or Mg^{2+} ions, and that it is reduced in sucrose-diluted cell-sap media. Therefore, the possibility cannot be entirely excluded that incorporation and transfer mechanisms are in some way obligatorily coupled to one another. It has been shown that amino acids are incorporated into microsome protein under the conditions used to study transfer of radioactivity.

The existence of a delay in the appearance of label in soluble protein presents an obvious analogy to the delay in the appearance of label in non-particulate specific proteins such as albumin (e.g. Peters, 1957), but there is as yet no evidence to show that the two phenomena are in fact related. The data reported by Peters are not adequate enough to establish clearly whether the lag period in the appearance of serum albumin in the medium is due merely to delay in excretion from the cell or in part due to the time required for release of albumin from the microsomes. The present results suggest that the latter might play some part, but it is unlikely that an adequate knowledge of this aspect of amino acid incorporation in cell-free systems will be obtained unless the labelling of specific proteins can be studied.

While this study was in progress, a number of reports appeared indicating that processes analogous to the one studied in the present investigation occur in other systems. Hendler (1957) reported that, if oviduct tissue was incubated with [^{14}C]amino acids and then homogenized, the ribonucleoprotein-containing cell debris so obtained appeared to release soluble radioactive protein. The transfer of radioactivity, which required ATP, took place under conditions in which there was no incorporation of amino acid into the ribonucleoprotein, and the radioactivity was present in protein fractions normally found in the cell sap. Rabinovitz & Olson (1957) also briefly reported similar results for reticulocytes; in this case the labelling of soluble haemoglobin was studied. Thus ribonucleoprotein labelled in intact cells can subsequently release soluble proteins under cell-free conditions, also by an energy-requiring process.

Campbell, Greengard & Kernot (1958) have recently reported that an albumin fraction bound to microsomes becomes labelled upon incubation of a microsome–cell-sap system from regenerating rat liver, but so far they have not reported the presence of label in albumin present in the cell sap. A failure to demonstrate the presence of labelled albumin in cell sap might be explained by interference with dissociation of albumin from microsome material resulting from cell damage. However, the studies of Hendler (1957) and Rabinovitz & Olson (1957) indicate that soluble proteins can be dissociated from ribonucleoprotein under cell-free conditions, at least with other tissues. The fact that in microsome–cell-sap systems there is an appreciable amount of label in cell-sap protein as a whole, but not in albumin, may indicate that it is not dissociation of protein from the microsomes that is interfered with, but rather that there is interference with the synthesis of specific proteins. Cell damage may disturb the normal pattern of protein synthesis; the differences in turnover of the protein of subfractions of microsomes labelled *in vivo* and in the cell-free system reported previously (Simkin & Work, 1957*b*) could be a reflexion of this. Thus while specific protein, such as albumin, might still be synthesized to some extent, protein synthesis might largely be redirected towards the synthesis of some non-specific or in-

complete protein. The failure to detect labelled albumin could, however, be the result of very great dilution with the pool of unlabelled albumin present in the system. It is hoped that the results of an investigation now in progress into the nature of the soluble protein labelled in the microsome–cell-sap system will clarify this point.

SUMMARY

1. When a microsome–cell-sap preparation from guinea-pig liver is incubated together with 3-phosphoglycerate, an adenosine triphosphate generator, and Mg^{2+} ions, there is incorporation of [^{14}C]amino acids into the protein of both microsome and cell-sap fractions. There is a delay of about 10 min. before appreciable label appears in the protein of the cell sap.

2. There is no significant incorporation under a variety of conditions of [^{14}C]amino acids into the protein of the cell sap in the absence of microsome material.

3. When microsome material is labelled by incubation with [^{14}C]amino acids for 10 min., then reincubated with fresh unlabelled cell sap, there is an increase in the specific activity of the cell-sap protein when the system is supplemented with 3-phosphoglycerate and Mg^{2+} ions; this is accompanied by an approximately equivalent decrease in the radioactivity of the microsome protein. The evidence obtained suggests that there is transfer of radioactivity from microsome to cell-sap protein.

4. In the absence of 3-phosphoglycerate, there is a marked reduction in the extent of labelling of cell-sap protein, indicating that an adenosine triphosphate-dependent reaction is associated with the transfer of radioactivity from microsome material. Absence of Mg^{2+} ions also reduces the labelling of cell-sap protein. Replacement of 75% of the cell sap by 0·25 M-sucrose reduces the transfer of radioactivity from labelled microsomes, despite supplementation with adenosine triphosphate and guanosine triphosphate.

5. Microsome material labelled by incubation with [^{14}C]amino acids for 10 min. is still able to incorporate amino acids into its protein when resuspended in fresh cell sap, although at a somewhat reduced rate.

6. The labelled material present in the cell sap appears to be largely in the form of soluble protein, and the possible nature of this material is discussed.

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The Relationship of the Tricarboxylic Acid Cycle to the Synthesis of δ-Aminolaevulic Acid in Avian Erythrocyte Preparations

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Shemin & Russell (1953) showed conclusively by ^{15}N- and ^{14}C-labelling experiments that δ-aminolaevulic acid incubated with duck blood was incorporated into the haem formed. Confirmation of δ-aminolaevulic acid as a porphyrin precursor in lysed red-cell systems was forthcoming from Neuberger & Scott (1953) and Dresel & Falk (1953).

It was postulated by Shemin & Kumin (1952) that succinyl-coenzyme A was involved in porphyrin synthesis and, upon the recognition of δ-aminolaevulic acid as a porphyrin precursor, it was suggested that succinyl-coenzyme A coupled with glycine to form δ-aminolaevulic acid via the unstable α-amino-β-oxoadipic acid (Shemin & Russell, 1953).

The evidence for the involvement of succinyl-coenzyme A is that an unsymmetrical four-carbon unit closely related to succinate is concerned in porphyrin biosynthesis (Shemin & Kumin, 1952) and that coenzyme A stimulates the synthesis from succinate.

The present work was initiated in an attempt to provide more direct evidence for succinyl-coenzyme A being a precursor of δ-aminolaevulic acid and at the same time to explore more fully the relationships between the tricarboxylic acid cycle and the initial stages of porphyrin biosynthesis. A system prepared from normal chicken erythrocytes and capable of synthesizing efficiently δ-aminolaevulic acid *in vitro* has been employed for this purpose.

During the course of the investigations described here, two communications have appeared (Laver & Neuberger, 1957; Gibson, Laver & Neuberger, 1958) which describe work with systems prepared from the erythrocytes of hens rendered anaemic with phenylhydrazine. The utilization of succinyl-coenzyme A for δ-aminolaevulic acid synthesis has been demonstrated in these systems.

EXPERIMENTAL

Materials

The blood used was collected from normal adult domestic fowls (*Gallus domesticus*) pooled for marketing. Collection was made into a polythene bottle containing penicillin G, streptomycin and heparin as described by Dresel & Falk (1954).

Citric acid (A.R.), fumaric acid, succinic acid and malic acid were obtained from British Drug Houses Ltd. Fumaric and succinic acids were recrystallized as sodium salts from aqueous ethanol. Malic acid was recrystallized from *iso*-butyl methyl ketone and chloroform (Montgomery & Webb, 1956*a*). Oxaloacetic acid, α-oxoglutaric acid and DL-*iso*-citric acid lactone were obtained from L. Light and Co. Ltd. The lactone was hydrolysed before use, as described by Krebs & Eggleston (1944). Adenosine triphosphate (ATP) and coenzyme A (CoA) were supplied by the Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. D-*iso*Citric acid lactone was a gift from Sir Hans Krebs, F.R.S., fluorocitric acid a gift from Sir Rudolph Peters, F.R.S., and α-lipoic acid a gift from Dr J. A. Elvidge. 5:6-Dimethylbenzimidazole was a gift from Glaxo Ltd. (through the good offices of Dr T. F. Macrae). Parapyruvate (γ-hydroxy-γ-methyl-α-oxoglutaric acid) was a gift from Dr H. Heath.

Methods

Preparation of tissue system. Chick erythrocytes were washed, lysed and restored to isotonicity as described by Dresel & Falk (1956). After centrifuging at about 1800 *g* for 15 min., the supernatant from the haemolysate was discarded and the precipitate was washed three times by resuspension in 0·9% NaCl followed each time by recentrifuging. The volume of the washed cell debris was then measured and to each 4 ml. was added 1 ml. of 0·9% KCl. The preparation was stirred well to ensure homogeneity and stored on crushed ice. All steps in the above procedure were carried out at about 4°. The preparation so obtained contained all the succinic-oxidase activity (as assayed in the Warburg respirometer) of the original haemolysate and synthesized δ-aminolaevulic acid (ALA) from glycine and succinate without concomitant production of detectable amounts of porphobilinogen or porphyrins.

Incubation. Incubation was conducted in 50 ml. Erlenmeyer flasks at 38° with shaking at about 100 shakes/min. Except where otherwise stated, 2 ml. of preparation was incubated in each flask in the presence of 0·5 ml. of 0·5 M-$MgCl_2$, 0·05 ml. of 5 mM-pyridoxal 5′-phosphate, 1·2 ml. of 0·1 M-phosphate buffer (pH 6·8) and 0·9% (w/v) KCl to bring the total volume after the addition of substrates, co-factors, etc. to 5 ml.

Identification and estimation of δ-aminolaevulic acid. ALA was identified by paper chromatography after prior conversion into 4-2′-carboxyethyl-3-ethoxycarbonyl-2-methylpyrrole by the method of Mauzerall & Granick (1956). The pyrrole was extracted with ether and chromatographed alongside, and as a mixed sampled with, a specimen of the pyrrole prepared from authentic ALA. Ascending chromatograms were run on Whatman no. 1 paper with ammonia (sp.gr. 0·880)–water–butanol (2:98:100, by vol.). The pyrrole was detected as a pink spot (R_F 0·40) on spraying the paper with the *p*-dimethylaminobenzaldehyde reagent described by Mauzerall & Granick (1956).

For estimation of ALA the reaction mixture (5 ml.) was partially deproteinized by treatment with 0·5 ml. of 10% (w/v) solution of $ZnSO_4,7H_2O$. After centrifuging, Mauzerall & Granick's (1956) procedure was applied to a measured volume of the supernatant, small quantities of residual protein brought down during heating being removed by recentrifuging.

That the material estimated as ALA was in fact this substance was verified by the chromatographic technique described each time a new substrate or addition was made to the incubate.

Identification of keto acids. Keto acids were identified by paper chromatography of their 2:4-dinitrophenylhydrazones as described by Cavallini, Frontali & Toschi (1949*a*, *b*).

Identification of hydroxamates. Hydroxamates were separated and identified by the paper-chromatographic method described by Stadtman & Barker (1950).

RESULTS

Glycine and succinate as substrates

Fig. 1 illustrates the relationship between production of ALA and time of incubation. It shows increasing synthesis which attains a plateau after a period of about 3 hr. Except where otherwise indicated, an incubation period of 3 hr. was used, therefore, in the ensuing experiments. The relatively marked loss of activity by the system on ageing can also be seen in Fig. 1.

The effect of glycine concentration on synthesis of ALA is shown in Fig. 2. The curve does not pass through the origin because of the presence of a small mount of endogenous glycine. Maximal synthesis was obtained with glycine concentrations of the order of 30 mM.

The relationship between succinate concentration and the production of ALA under the standard conditions of incubation and glycine concentration is illustrated in Fig. 3, maximal effects being observed with succinate levels of about 10 mM. The enzyme system was fully saturated when these substrates were present in the concentrations indicated.

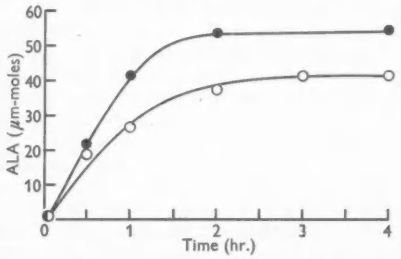

Fig. 1. Effect of duration of incubation on ALA synthesis. ●, Fresh preparation; ○, preparation which had been stored on crushed ice for 24 hr. before use. Preparations contained 25 mM-succinate and 30 mM-glycine, and were incubated under the standard conditions described in the text.

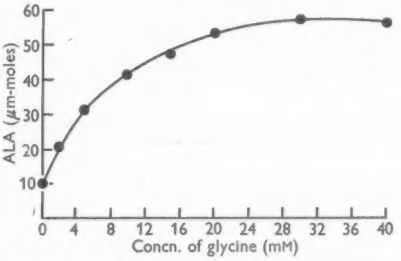

Fig. 2. Effect of glycine concentration on formation of ALA. Succinate was present in a concentration of 25 mM. Incubations were conducted under standard conditions.

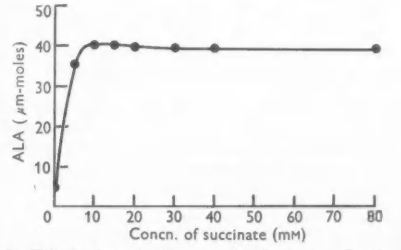

Fig. 3. Effect of succinate concentration on production of ALA. Glycine was present in a concentration of 30 mM. Incubations were conducted under standard conditions.

Glycine and other tricarboxylic cycle intermediates as substrates

Various intermediates of the tricarboxylic acid cycle at molar concentrations comparable to that found to give maximal synthesis of ALA from succinate (i.e. 10 mM) were examined for capacity to replace succinate in the system. The results of this preliminary screening are shown in Table 1. They indicate vast superiority of citrate and *iso*-citrate over either α-oxoglutarate or succinate as substrate for synthesis of ALA. Fumarate, malate and oxaloacetate had very little activity in this respect. The small but detectable yield of ALA obtained without added substrate is due to traces of endogenous substrate in the preparation (see also Fig. 3).

More detailed examination of citrate, *iso*citrate and α-oxoglutarate, at various concentrations, as alternative substrates to succinate was made and the results are recorded in Fig. 4. It is noted that excess of these substrates is inhibitory.

Table 1. *Comparison of various tricarboxylic acid cycle intermediates as substrates for the synthesis of δ-aminolaevulic acid*

Incubations were conducted under standard conditions (see under Methods) with 30 mM-glycine. All substrates were examined at a concentration of 25 mM. No change in pH was observed after incubation with any of the substrates.

Substrate	ALA (μm-moles)
Citrate	181
D-*iso*Citrate	198
α-Oxoglutarate	93
Succinate	68
Fumarate	6
Malate	10
Oxaloacetate	21
None added	4

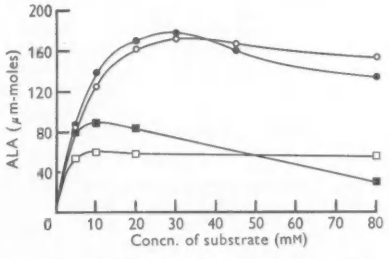

Fig. 4. Comparison of citrate, D-*iso*citrate and α-oxoglutarate with succinate as substrates for synthesis of ALA. ●, Citrate; ○, D-*iso*citrate; ■, α-oxoglutarate; □, succinate. Incubations were conducted under standard conditions in the presence of 30 mM-glycine.

Paper chromatography of the above incubates for identification of keto acids showed the formation of α-oxoglutarate from citrate, *iso*citrate and succinate although with succinate the amount, as judged visually from the fluorescence of the dinitrophenylhydrazones in ultraviolet light, was very small compared with that formed from the two other substrates.

Effect of various co-factors. Pyridoxal 5′-phospate (50 μM) was added to the incubates as a matter of routine throughout the present work as a result of the report of Schulman & Richert (1956) that it is involved in the early stages of porphyrin synthesis before the formation of ALA. Omitting it from the system caused a decrease of approximately 40% in ALA synthesis, as can be seen from Table 2.

Other possible co-factors examined for effect on ALA synthesis by the present preparation were CoA, ATP, α-lipoic acid, thiamine pyrophosphate and the ribosides adenosine, guanosine and inosine. The results are shown in Table 2. CoA was found to stimulate ALA synthesis from each of the three tricarboxylic acid cycle intermediates, *iso*citrate, α-oxoglutarate and succinate. ATP, however, exerted a stimulatory influence only when succinate was the substrate and produced slight but reproducible inhibition when either *iso*citrate or α-oxoglutarate was being utilized for the production of ALA. α-Lipoic acid produced an increase in ALA yield from *iso*citrate or α-oxoglutarate but had little effect on that from succinate. Neither thiamine pyrophosphate nor the ribosides had any significant influence on ALA synthesis from any of the three substrates examined.

Inhibitors. Fluorocitrate, a known inhibitor of aconitase (Morrison & Peters, 1954), was examined for effect on ALA synthesis from each of the four tricarboxylic acid cycle intermediates, citrate, *iso*citrate, α-oxoglutarate and succinate. The results recorded in Table 3 show, as would be expected if the tricarboxylic acid cycle is the main pathway by which a C_4 unit is provided for ALA synthesis, a marked inhibition of ALA synthesis from citrate but not from the other three substrates.

Another inhibitor examined was parapyruvate (γ-hydroxy-γ-methyl-α-oxoglutaric acid). This substrate was found by Montgomery & Webb (1956*b*) and Montgomery, Fairhurst & Webb (1956) to be an efficient and specific inhibitor of the oxidative decarboxylation of α-oxoglutarate by guinea-pig-heart mitochondria. The results with this material, shown in Table 3, indicate a suppression of ALA formation from citrate, *iso*citrate and α-oxoglutarate but not from succinate.

Table 2. *Effect of possible co-factors on the synthesis of δ-aminolaevulic acid from* isocitrate, *α-oxoglutarate or succinate*

Standard incubation procedure was followed with 30 mM-glycine, 25 mM-α-oxoglutarate, 25 mM-succinate and 80 mM-DL-*iso*citrate (experimentally found to be equivalent, in synthesis of δ-aminolaevulic acid by this preparation, to 25 mM-D-*iso*citrate).

Additions	ALA (μm-moles)		
	*iso*Citrate	α-Oxoglutarate	Succinate
None	144	76	58
None (pyridoxal 5′-phosphate omitted)	82	43	33
ATP (0·5 mM)	124	48	164
CoA (0·5 mM)	218	114	146
Thiamine pyrophosphate (mM)	126	70	54
α-Lipoic acid (mM)	216	91	60
Adenosine (mM)	145	76	58
Guanosine (mM)	140	72	52
Inosine (mM)	145	72	57

Table 3. *Effect of fluorocitrate and parapyruvate on δ-aminolaevulic acid formation*

After pre-incubation of inhibitors and preparation for 15 min. under standard conditions plus 30 mM-glycine, substrates were added and the incubation conducted for a further 3 hr. Citrate, α-oxoglutarate and succinate were used in a concentration of 25 mM and DL-*iso*citrate was employed at a concentration equivalent to 25 mM-D-*iso*citrate. Fluorocitrate was 0·23 mM and parapyruvate 4 mM. Percentage inhibitions are shown in parentheses.

Inhibitor	ALA (μm-moles)			
	Citrate	*iso*Citrate	α-Oxoglutarate	Succinate
None	121	126	55	41
Fluorocitrate	48 (60%)	124 (1%)	55 (0%)	40 (2%)
Parapyruvate	16 (87%)	15 (88%)	12 (78%)	45 (0%)

Antagonistic effect of succinate on the utilization of citrate and isocitrate

In an experiment designed to test the effect of simultaneous addition of more than one substrate to the system, it was observed that ALA formation from succinate and *iso*citrate together was considerably less than that obtained in a control incubate containing *iso*citrate alone.

More detailed examination of this effect indicated that increasing concentrations of succinate progressively depressed ALA synthesis until a level was attained comparable with the maximal yield obtained with succinate alone. Further, it was found that the inhibition could be reversed by increasing the *iso*citrate concentration although, as can be seen from Fig. 4, this substrate was already at its optimum concentration for ALA synthesis. The results of a typical experiment are depicted in Fig. 5.

This inhibitory effect of succinate on ALA formation could also be demonstrated when either citrate or α-oxoglutarate was the second substrate. In the latter case, however, the inhibition was potentiated and not reversed by increasing the concentration of the second substrate (α-oxoglutarate). This is shown in Fig. 6.

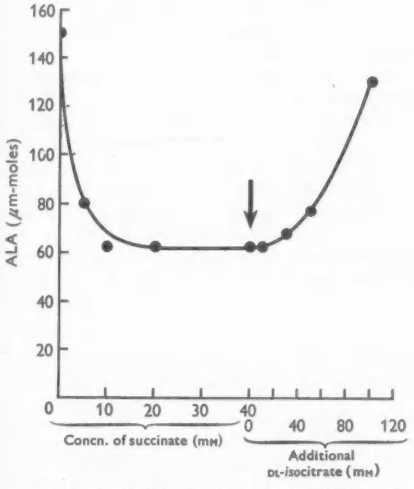

Fig. 5. Succinate inhibition of synthesis of ALA from *iso*citrate and its reversal by increasing concentrations of *iso*citrate. DL-*iso*Citrate, prepared by hydrolysis of the lactone, was present initially at its optimum concentration for ALA synthesis (equivalent to 30 mM-D-*iso*citrate). At the point indicated by the arrow increasing amounts of the DL-*iso*citrate preparation were added. Incubations were conducted under standard conditions in the presence of 30 mM-glycine.

The depression of ALA synthesis from citrate by addition of succinate could not be alleviated by CoA or α-lipoic acid (Table 4). ATP, however, did relieve the inhibition to a slight extent.

Nature of the inhibitory effect of succinate. In order to elucidate the mode of action of succinate as an inhibitor of ALA synthesis from citrate, *iso*citrate or α-oxoglutarate, other tricarboxylic acid cycle intermediates were examined for similar effects. The primary product of succinate oxidation in the tricarboxylic acid cycle is fumarate, and at equimolar concentration it was found to be even

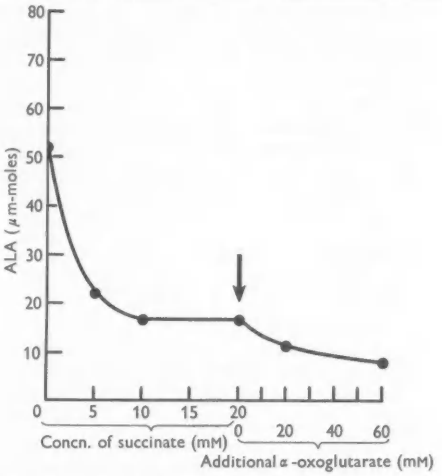

Fig. 6. Succinate inhibition of synthesis of ALA from α-oxoglutarate and its potentiation by increasing amounts of α-oxoglutarate. Incubations were conducted under standard conditions in the presence of 30 mM-glycine.

Table 4. *Inhibition by succinate of synthesis of δ-aminolaevulic acid from citrate in presence of co-factors*

Incubations were carried out under standard conditions with glycine and citrate, both at a concentration of 30 mM. Succinate was used at 10 mM. CoA and ATP were employed at concentrations of 0·5 mM, α-lipoic acid at 10 mM and thiamine pyrophosphate (TPP) at 10 mM.

Additions	ALA (μm-moles)	Inhibition (%)
None	149	66
Succinate	50	
CoA	311	65
Succinate + CoA	109	
ATP	140	40
Succinate + ATP	84	
α-Lipoic acid + TPP	215	62
Succinate + α-lipoic acid + TPP	82	

more efficient than succinate in depressing ALA formation. Malonate (10 mM) was found to alleviate the inhibition by succinate to a significant extent. The results are recorded in Table 5.

The observations were interpreted as indicating that succinate exerted its depressant effect on the synthesis of ALA only after prior conversion into fumarate. Consequently, other tricarboxylic acid cycle intermediates lying after fumarate in the oxidative direction of the cycle were also investigated. The results, shown in Table 6, show that malate and oxaloacetate also depress ALA formation from citrate more efficiently than does succinate, although not to as low a level as does fumarate.

Table 5. *Comparison of fumarate and succinate as inhibitors of the synthesis of δ-aminolaevulic acid from citrate and the effect of malonate on the inhibitions*

Incubations were carried out under standard conditions with glycine and citrate both at a concentration of 30 mM. Succinate, fumarate and malonate were used at a level of 10 mM.

Additions	ALA (μm-moles)	Inhibition (%)
None	94	—
Succinate	38	60
Fumarate	19	80
Succinate + malonate	54	42
Malonate	66	30

Table 6. *Comparison of malate, oxaloacetate and acetate with succinate and fumarate as inhibitors of the formation of δ-aminolaevulic acid from citrate*

All incubates contained 30 mM-glycine and 25 mM-citrate. Incubation was under standard conditions. All additions were made at a level of 25 mM.

Additions	ALA (μm-moles)	Inhibition (%)
None	195	—
Succinate	80	59
Fumarate	4	98
Malate	26	87
Oxaloacetate	24	88
Acetate	198	0

Formation of succinyl-coenzyme A and synthesis of δ-aminolaevulic acid

By using similar incubation conditions to those described in the Methods section, but omitting glycine and pyridoxal phosphate, acyl-CoA derivatives were trapped with hydroxylamine and estimated by the method of Lipmann & Tuttle (1945). The formation of ALA was determined in parallel incubations by using the complete system. The results are shown in Table 7. Paper-chromatographic checks on the hydroxamates formed, by the method of Stadtman & Barker (1950), showed that the only detectable product from these incubates was succinohydroxamic acid.

The results show that α-oxoglutarate is a much better substrate for succinyl-CoA formation than was either citrate or succinate, as was to be expected if succinyl-CoA was produced directly from α-oxoglutarate in the tricarboxylic acid cycle. However, α-oxoglutarate is a less efficient substrate than is citrate (Tables 1 and 7) for the synthesis of ALA; the reasons for this are discussed below. Table 7 also indicates that fumarate must inhibit the synthesis of ALA from citrate at some step before the formation of succinyl-CoA.

Effect of 5:6-dimethylbenzimidazole. Abbott & Dodson (1954) showed that 5:6-dimethylbenzimidazole inhibited the incorporation of [^{15}N]glycine into haem by chicken-erythrocyte preparations and that this could be traced to an inhibition at a level before the formation of ALA in porphyrin synthesis (Abbott, Dodson & Auvil, 1956).

5:6-Dimethylbenzimidazole was examined in the present system at a concentration (7·5 mM) which had been previously found to give maximal inhibition. The results, illustrated in Table 7, show that 5:6-dimethylbenzimidazole produces an apparent

Table 7. *Comparison of the formation of succinyl-coenzyme A and δ-aminolaevulic acid under various conditions*

Preparations for ALA assay were incubated under the standard conditions with 30 mM-glycine. Citrate, α-oxoglutarate and succinate were 30 mM, fumarate 10 mM and 5:6-dimethylbenzimidazole 7·5 mM. Incubations for hydroxamate estimation were similar, but pyridoxal phosphate was omitted and hydroxylamine (40 mM, pH 7·0) replaced glycine.

Substrate	Additions	Succino-hydroxamic acid (μm-moles)	ALA (μm-moles)
Citrate	—	410	134
Citrate	Fumarate	230	6
Citrate	5:6-Dimethylbenzimidazole	550	27
Citrate	α-Oxoglutarate	610	99
α-Oxoglutarate	—	650	60
Succinate	—	270	45
Succinate	5:6-Dimethylbenzimidazole	540	10

increase in succinyl-CoA formation and yet inhibits ALA synthesis by about 80%. The implications of this are discussed below.

Table 8 shows the result of increasing the glycine and pyridoxal 5′-phosphate concentrations in systems in which ALA synthesis from citrate was partly inhibited by 5:6-dimethylbenzimidazole. The inhibition was apparently relieved to some extent by increasing the glycine concentration, but not by increasing the amount of pyridoxal 5′-phosphate present.

DISCUSSION

For the purposes of the present investigation, an enzyme system was desired which would synthesize ALA in measurable quantity but in which synthesis would not proceed further. In addition, it was considered highly desirable that such a system should originate from normal animals, since intoxication with drugs such as phenylhydrazine could conceivably impair or disorganize the tricarboxylic acid cycle (cf. Sprinz & Waldschmidt-Leitz, 1953; Geber & Rostorfer, 1954) the relation of which to ALA synthesis it was desired to investigate. A suitable system was found in the fowl-erythrocyte preparation described here.

Examination of various tricarboxylic acid cycle intermediates (Table 1, Fig. 1) showed that the following descending order of efficiency as substrates for ALA synthesis exists in this system: *iso*citrate, citrate, α-oxoglutarate, succinate, oxaloacetate, malate, fumarate. Citrate is apparently utilized for ALA synthesis after prior conversion into *iso*citrate, as was shown (Table 3) by the use of the specific inhibitor of aconitase, namely fluorocitrate.

*iso*Citrate appears to be converted into α-oxoglutarate before the formation of ALA, as has been indicated by: (i) paper-chromatographic identification of keto acids formed; (ii) the inhibition of the synthesis of ALA from these two substrates by parapyruvate, a specific inhibitor of the oxidative decarboxylation of α-oxoglutaric acid (Table 3); and (iii) the stimulation of the formation of ALA from these substrates by α-lipoic acid, an essential component of the α-oxoglutaric oxidative decarboxylation system (Table 2). These interconversions are all consistent with the presence of an active tricarboxylic acid cycle in the present system.

Confirmation of the involvement of CoA in the synthesis of ALA was obtained by the large stimulatory capacity of this co-factor on all substrates examined. ATP stimulated the formation of ALA from succinate, but not from *iso*citrate or α-oxoglutarate, with which, on the contrary, it produced slight but reproducible inhibitions (Table 2). These observations are in agreement with the direct formation of succinyl-CoA from succinate (requiring ATP) as well as from α-oxoglutarate (not requiring ATP) according to the scheme (cf. Shemin & Kumin, 1952; Wriston, Lack & Shemin, 1955; Kaufman, Gilvarg, Cori & Ochoa, 1953):

$$\alpha\text{-oxoglutarate} \xrightarrow[\text{etc.}]{+\text{CoA}} \text{succinyl-CoA} \xrightarrow[+\text{CoA}]{+\text{ATP}} \text{succinate}$$

Production of succinyl-CoA from citrate, *iso*citrate or α-oxoglutarate would, however, generate ATP without requiring it at any stage; hence it seems that a relatively large exogenous pool of ATP might depress the formation of succinyl-CoA from these substrates. An analogous situation has been reported by Sanadi & Littlefield (1951) in the inhibition by added ATP of the succinylation of sulphanilamide, when using a coupled α-oxoglutaric 'dehydrogenase' and pigeon-liver acylating system.

The inhibition by succinate of the synthesis of ALA from citrate or *iso*citrate brought the ALA level down to that obtained by using succinate alone (Figs. 4, 5). This confirms the existence of two pathways for succinyl-CoA formation: (i) from α-oxoglutarate, and (ii) from succinate directly; and it is compatible with the scheme shown above.

The inhibitory effect of succinate has been shown (Tables 5, 6) to be attributable not to succinate but to the formation of fumarate, malate or oxaloacetate. The yields of ALA from citrate in the presence of each of these acids appear to be influenced by two complimentary factors: the inhibitory effect and the partial utilization of these inhibitory acids as substrates (compare Tables 1 and 6). This explains why ATP appears to alleviate the succinate inhibition to some degree, for it has already been shown that this co-factor stimulates the utilization of succinate for the synthesis of ALA (Table 4).

Table 8. *Effect of increasing the glycine and pyridoxal 5′-phosphate concentrations on the inhibition of synthesis of δ-aminolaevulic acid by* 5:6-*dimethylbenzimidazole*

Incubations were under standard conditions. 5:6-Dimethylbenzimidazole (DMB) was used at a concentration of 7·5 mM. Citrate (30 mM) was used throughout as substrate.

Additions	ALA (μm-moles)
Glycine (30 mM)	84
Glycine (30 mM) + DMB	4
Glycine (30 mM) + DMB + pyridoxal 5′-phosphate to a final concentration of 0·5 mM	3
Glycine (0·1 M)	78
Glycine (0·1 M) + DMB	24

If allowance is made for utilization of these dicarboxylic acids as substrates, it appears, by comparing Tables 1 and 6, that oxaloacetate is probably as effective as fumarate in inhibiting the formation of ALA from citrate. Further, as the tricarboxylic acid cycle is very active in the oxidative direction in this preparation, it would seem most likely that, of fumarate, malate and oxaloacetate, the last is the real inhibitor and that the other two acids mediate their effects only after oxidation to oxaloacetate.

The inhibitory effect of these dicarboxylic acids on the synthesis of ALA from citrate appears to be due to a suppression of the oxidative decarboxylation of α-oxoglutaric acid. Evidence for this is: (i) utilization of α-oxoglutarate for the formation of ALA is also inhibited by these acids (Fig. 6); (ii) the formation of succinyl-CoA from citrate is inhibited in the system in their presence (Table 7), i.e. the inhibition is between α-oxoglutarate and formation of succinyl-CoA. In speculating on purely structural grounds, it might be suggested that oxaloacetate competes in some way with α-oxoglutarate in the oxidative decarboxylation system.

The inhibition does not appear to be relieved by CoA or α-lipoic acid. It can, however, be completely reversed by increasing the citrate concentration (Fig. 5) and hence, by inference, the endogenous α-oxoglutarate concentration. However, this is not the same as adding α-oxoglutarate exogenously, as can be seen from Fig. 6; this is further discussed below.

In the case of succinate-inhibition of the synthesis of ALA from added α-oxoglutarate, the inhibition cannot be relieved by increasing the substrate concentration (Fig. 6). The reason for this can be seen in Table 7, for, although α-oxoglutarate is the most efficient of the substrates examined for the production of succinyl-CoA, it is much less efficient than either citrate or *iso*citrate as a substrate for ALA synthesis. This means that α-oxoglutarate is itself inhibiting the condensation of succinyl-CoA and glycine to form ALA.

The last-mentioned observation affords an explanation for some other apparently anomalous results. First, it explains the marked inhibition of the synthesis of ALA when excess of citrate, *iso*citrate or α-oxoglutarate is used (Fig. 4). Secondly, it shows why the production of ALA from α-oxoglutarate is less than that from citrate; for in the latter case α-oxoglutarate would be generated enzymically in a steady trickle and never attain significantly inhibitory concentrations, as when α-oxoglutarate is supplied exogenously.

5:6-Dimethylbenzimidazole appears, from Table 7, also to be exerting its inhibitory effect on the synthesis of ALA at the condensation step between succinyl-CoA and glycine. The reasons for this are not obvious, but the fact that the inhibition can be partly relieved by increasing the glycine concentration (Table 8) suggests that 5:6-dimethylbenzimidazole might act in suppressing activation of glycine by pyridoxal phosphate for the condensation. This postulation would explain the apparent increase in succinyl-CoA formation in the presence of 5:6-dimethylbenzimidazole, for without inhibition some succinyl-CoA would be drawn off to form ALA by combination with the small but significant amount of endogenous glycine in the preparation (see Fig. 1), with consequent decrease in the amount of succinyl-CoA detectable as hydroxamate.

Because of the observation that not all succinyl-CoA produced is trapped as hydroxamate, no attempt has been made to calculate the efficiency of the conversion of succinyl-CoA into ALA from the figures in Table 7.

The demonstration, in the present system, of antagonisms and inhibitions in the synthesis of ALA by normal tissue metabolites poses the interesting question whether these factors play a role in co-ordinating cellular metabolism, particularly in the biogenesis of ALA and its further utilization ('succinate–glycine cycle', porphyrin synthesis, etc.). In the present experiments these antagonisms and inhibitions result in drastic suppression of the synthesis of ALA, but, with more nearly physiological concentrations such as exist *in vivo*, they may well represent mechanisms by which the synthesis is normally regulated.

Further work upon this problem is in progress, with particular reference to the mechanism of inhibition by 5:6-dimethylbenzimidazole.

SUMMARY

1. A preparation of lysed normal chick erythrocytes has been described which actively synthesized δ-aminolaevulic acid from glycine in the presence of various tricarboxylic acid cycle intermediates, but which does not produce porphobilinogen or porphyrins.

2. The tricarboxylic acid cycle intermediates fall into the following decreasing order of effectiveness in δ-aminolaevulic acid synthesis: *iso*citrate, citrate, α-oxoglutarate, succinate, oxaloacetate, malate and fumarate.

3. Pyridoxal 5′-phosphate and coenzyme A stimulate the synthesis from all substrates examined. Adenosine triphosphate stimulates the production of δ-aminolaevulic acid from succinate but slightly inhibits that from citrate, *iso*citrate and α-oxoglutarate. α-Lipoic acid enhances synthesis from citrate, *iso*citrate and α-oxoglutarate, but not from succinate.

4. Use of the inhibitors fluorocitrate and parapyruvate shows that the interconversion of substrates before the formation of δ-aminolaevulic acid is consistent with an active tricarboxylic acid cycle.

5. Succinate inhibits the synthesis of δ-aminolaevulic acid by suppressing the formation of succinyl-coenzyme A from citrate, *iso*citrate or α-oxoglutarate after itself being first converted into fumarate, malate or oxaloacetate. The inhibition is reversed by increasing concentrations of citrate or *iso*citrate, but is potentiated by α-oxoglutarate.

6. α-Oxoglutarate added exogenously inhibits the condensation of glycine and succinyl-coenzyme A in the formation of δ-aminolaevulic acid.

7. 5:6-Dimethylbenzimidazole also inhibits the condensation of glycine and succinyl-coenzyme A. The inhibition here is partly relieved by increasing concentrations of glycine, but not by increasing concentrations of pyridoxal 5′-phosphate.

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Bound Amino Acids in Protein-free Extracts of Italian Ryegrass

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Protein-free diffusates from ryegrass were studied by Synge (1951), who showed that, whilst the greater part of the nitrogen is present as free amino acids, a substantial proportion (usually about 5% of the nitrogen of the diffusates) could be segregated in the form of chemically bound amino acids which could be set free by acid hydrolysis. These fractions of diffusate, obtained by electrical transport in a diaphragm cell, contained also much carbohydrate; their nitrogen content was not usually much more than 0·1% on dry matter. In the present paper we describe subsequent experiments aimed at characterizing more closely the forms in which bound amino acids occur in diffusates from grass. We have worked chiefly with Italian ryegrass (*Lolium multiflorum* Lam.), but have not found any essential differences from results previously obtained with perennial ryegrass (*L. perenne* L.). A few isolated experiments with diffusates from such diverse leaves as those of clover, raspberry, nettle, cabbage and potato showed that by the diaphragm-cell procedure

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qualitatively and quantitatively similar bound amino acid fractions could be obtained in each case.

In our first attempts to remove carbohydrates we found that the greater part of the bound amino acids could be adsorbed on charcoal (cf. Virtanen & Miettinen, 1953). From this the bulk of the carbohydrate was readily eluted by water, especially if it had been subjected to preliminary mild acid hydrolysis sufficient to break down fructosans and sucrose to monosaccharides. However, only very poor recoveries of bound amino acids resulted on eluting the charcoal with solutions of volatile displacing agents such as ethanol, butanol or phenol, even when the charcoal had been pretreated with stearic acid. Attempts at liquid–liquid extraction of the bound amino acids from water into such solvents as chloroform, ethyl acetate and butan-2-one likewise gave poor yields of bound amino acids, although simple carbohydrates were largely eliminated from such material as was extracted.

We then found that liquid–liquid extraction with phenol gave nearly complete extraction of the bound amino acids. Using a simple five-funnel countercurrent distribution we obtained from the crude diffusate an extract practically free from mono- and poly-saccharides, salts and the more hydrophilic free amino acids. This procedure has been briefly described (Synge & Wood, 1954) and has served as the fundamental step in all our subsequent work.

We subjected the material obtained by extraction into phenol to liquid–liquid chromatography on kieselguhr with successive use of the systems water–phenol and butanol–acetic acid–water. An extremely complicated set of fractions resulted, most of which on acid hydrolysis yielded much the same mixture of amino acids as that present in the starting material. Rechromatography of individual fractions gave reasonably sharp peaks, so we were forced to conclude that we were dealing with a very complicated mixture of substances.

At this stage we obtained evidence from a number of directions that the majority of the materials which we were studying were acidic in nature. We had considered at first that they were neutral compounds on account of their failure to migrate out of the specimen compartment of the four-compartment diaphragm cell when its contents were maintained at pH 6 (Synge, 1951). This apparent contradiction was resolved in the course of some work on electrical transport inside membranes (Synge, 1957), from which it became apparent that materials in our fractions were probably strongly adsorbed on the formolized parchment membrane, thus influencing its electrochemical behaviour in such a way as to restrict its capacity for transmitting at least the less mobile anions (cf. Fridrikhsberg & Gutman, 1953). The implication is that much of the material which is anionic at pH 6 fails under these conditions to penetrate the membrane and that therefore some of the acidic compounds may be rather arbitrarily distributed between fractions 4-*N* and 4-*A* in the 4-compartment cell procedure of Synge (1951). Accordingly, in the work now reported we have eliminated this step from our fractionation procedures.

The knowledge that the compounds to be studied were mainly acids suggested additional techniques for their fractionation, particularly anion-exchange chromatography and zone electrophoresis. As we are dealing with a very complicated mixture, it has been necessary to devise procedures for the earlier steps in the fractionation which could be applied to relatively large amounts of material. Accordingly, the initial extraction into phenol has been followed by displacement chromatography on an anion-exchange resin. The fractions resulting have been subjected to zone electrophoresis in volatile buffer in silica jelly. This last procedure has some novel features; it has proved particularly useful in dealing with the present materials and may have more general applicability.

The fractions resulting from the zone electrophoreses have been small enough for convenient application of various chromatographic procedures involving elution development. So far, only one rather small fraction has been studied in detail in this way, with disconcertingly complicated results.

A particularly interesting feature of the anion-exchange chromatography has been the failure of the greater part of the bound amino acids to be retained on the more tightly cross-linked resins, despite the demonstrably anionic character of the material. On resin with a lower degree of cross-linking more of this material is retained, much of it irreversibly. We conclude that these substances have somewhat high molecular weights, despite their ability to pass through cellophan.

The simplest view of the chemical nature of these acidic components of the bound amino acids (see Discussion) would be that they are *N*-acyl derivatives of common amino acids (among which six or seven species predominate). The acyl groups seem to be those of a diversity of organic acids, probably many of them polybasic. The evidence as to conjugated oxalic acid is of interest in this connexion.

It is thus likely that the bulk of the bound amino acids in diffusates from Italian ryegrass, which accounts for about 5 % of the non-protein nitrogen of the grass, is not peptide in nature. However, the fractionation procedure which we have devised in the course of this work has also led to the segregation of a significant fraction (*PWPC*) for which the analytical results now published are consistent

Table 1. *Specimens of Italian ryegrass used for preparation of diffusates*

Specimen	Hour of cutting	Date of cutting	Total N as percentage of dry matter of grass	Whether frozen or fresh	Diffusible N: As percentage of total N of grass*	Diffusible N: As percentage of total N of expressed† juice	Diffusible N: In expressed juice (mg./ml.)
I	9–10	12. x. 51	2·7	Fresh	9·7	96	0·84
II	11–13	29. iv. 53	1·9	Fresh	10·4	98	0·58
III	10–11	27. vi. 53	1·5	Frozen	15·5	89	1·02
IV	15–16	12. x. 53	1·6	Frozen	21·0	84	1·27
V	14–15	6. x. 54	1·6	Frozen	24·8	97	1·10
VI	11–12	9. v. 56	3·7	Fresh	13·7	91	1·05
VII	9–11	6. ix. 56	3·4	Fresh	10·2	95	0·56
VIII	9–12	23. iv. 57	3·3	Fresh	10·1	89	0·64

* This was approximately calculated by assuming that the diffusible N of the juice squeezed out of the grass was present at the same concentration throughout the water of the specimen (as determined by loss of weight on drying a sample at 105°).

† For method of calculation see Synge (1951).

with the presence of genuine peptides. Mr P. R. Carnegie is at present studying this fraction in greater detail. We also hope in this Laboratory to trace the metabolic paths in the leaf of amino acid residues of the various fractions.

We were greatly helped in the preliminary stages of our work by Dr Nils Ellfolk, whose unpublished studies of grass diffusates helped us to recognize the anionic character of the bound amino acids. Dr Ellfolk also detected conjugated oxalic acid in various fractions from the diffusates. His work done here concerning the artifactual occurrence of pyrrolidonecarboxylic acid in grass has been published (Ellfolk & Synge, 1955).

MATERIAL

Italian ryegrass grown in the course of ordinary farming operations on the Duthie Experimental Stock Farm of this Institute was used at the pre-flowering or early flowering stage. All parts of the plant above about 1 in. from the ground were taken. Diffusates were obtained by ether treatment as described by Synge (1951), except that the expressed juice was usually concentrated about tenfold by evaporation *in vacuo* before dialysis, and that filtration was omitted. In some of our earlier preparations the grass had been frozen and stored at −20° before subjecting it to thawing and treatment with ether. Evaporation during cold storage led to the expressed juices being more concentrated, but the resulting diffusates were not obviously different from those from fresh grass in their behaviour during fractionation. Details of the original grasses and diffusates prepared from them are given in Table 1.

METHODS

Initial fractionation of diffusates

Fig. 1 shows the scheme for fractionation of the diffusates, developed after considerable trial and error, by which the bound amino acids (BAA) may be segregated into reproducible fractions; the nature of the end-fractions obtained is, as far as our experience goes, independent of the sequence in which the different operations are applied to the material; however, the scheme given in Fig. 1 has advantages for dealing with the amounts of material that must be handled at each step.

Countercurrent distribution in phenol–water (cf. Synge & Wood, 1954; Ellfolk & Synge, 1955). In a typical preparation, diffusate (from about 30 lb. of fresh grass) containing about 250 g. of dry matter and 3 g. of N was evaporated to small volume *in vacuo*, acidified to pH 2 (glass electrode) by addition of 12N-H_2SO_4 and transferred in water (final vol. approx. 300 ml.) to the first of a train of five separating funnels. Recently distilled A.R. phenol was made into two liquid phases by addition of water. A charge of 300 ml. of top (aqueous) phase was placed in each of funnels 2–5. A succession of 300 ml. charges of bottom (phenol-rich) phase was then passed through the train, equilibrating by shaking each funnel and allowing to settle before transferring the phenol-rich phase to the next funnel in the series. The aqueous phase in funnel 1 remained denser than the phenol-rich phase throughout the operation. In funnel 2 there was a tendency for the aqueous phase to become equal in density with the phenol-rich phase, and sometimes a little water was added to this funnel to increase the rate of settling of the phases. We were surprised to find how little emulsification occurred; whenever settling time was excessive, the funnel contents were removed and centrifuged. The first eight charges of phenol-rich phase emerging from the train were pooled (fraction *P*), as were the second eight (fraction *Q*). The aqueous residues remaining in the funnels were then pooled (fraction *R*) and usually discarded. The bulk of the BAA as well as of the brown pigment of the diffusate was taken off in fraction *P*. At the stage when this had emerged from the train, the aqueous phase in funnel 5 contained no SO_4^{2-}; small traces of monosaccharide were revealed in it by paper chromatography, but higher saccharides initially present in the diffusate were absent.

Chromatography on anion-exchange resin. The resin (500 g. of AG2-X10, 200–400 mesh/in. processed by Bio-Rad Laboratories, 800 Delaware, Berkeley, Calif., from Dowex 2-X10) was received in the chloride form, packed

into a column and washed with M-sodium acetate until the effluent was Cl^--free. The column was then washed through with 0·2M-sodium acetate; the resin was then removed as a slurry in this solvent and packed wet into three tubes (I, 25 cm. × 5·4 cm.; II, 16·7 cm. × 2·2 cm.; III, 10 cm. × 1·2 cm.) arranged so that they could be connected in series by narrow tubes for displacement development (cf. Claesson, 1947; Hagdahl, 1948; Partridge & Brimley, 1951).

Fraction *P* was concentrated by evaporation *in vacuo* with additions of water repeated as often as phenol-rich phase only was present until all phenol had been removed. The resulting brown solution, when concentrated, slowly deposited a little insoluble material, but much further precipitation occurred if the solution was diluted with water. The solution (containing approx. 25 g. of dry matter and 700 mg. of N) was brought to pH 6 (glass electrode) by cautious addition of 5N-NaOH (approx. 10 ml.). This decreased the tendency to precipitation. The solution (approx. 100 ml.) was then transferred to the top of column I; effluent fractions were collected from the bottom of column I at this stage. The solution was allowed to run into the column; the top centimetre of the column was stirred or air pressure was applied if the precipitate which collected on top of the column obstructed flow. Three successive 20 ml. portions of 0·2M-sodium acetate were used for washing the material into the column and development was then continued with the same solvent. Most of the pigment moved as a broad fast band (collected as fraction *PV*, about 200 ml.). Behind this, the column was slightly but uniformly stained with pigment, while substantial pigment was held in stationary brown zones at the top of the column. Development continued, giving fraction *PW* in about 2 l. of solvent, the latter portions of which were colourless and nearly free from N.

Then 0·2N-HCl was substituted for 0·2M-sodium acetate as the developing solvent, and columns II and III were coupled in series below column I. Fractions of suitable size were collected. The sodium acetate issuing from column III was soon replaced by acetic acid (resulting from displacement of acetate by chloride from the resin as the HCl front travelled down the column). Fractions eluted by this solvent are referred to collectively as *PX*. The HCl front was preceded by displacement zones of a number of organic acids, accompanying which was a variable and complicated set of narrow zones of reddish pigment which gave useful information about the effectiveness of the front-straightening action of columns II and III. Smaller fractions were collected when the displacement zones began to emerge (fraction group *PY*) and larger fractions again after break-through of the chloride front (fraction group *PZ*, eluted in about 1 l. of solvent).

Further fractionation of PV and PW. Materials in fraction groups *PX*, *PY* and *PZ* could be obtained in form suitable for analysis or further fractionation by simple evaporation. However, those in fractions *PV* and *PW* were dissolved in aqueous sodium acetate. To free them from salt they were concentrated to small volume *in vacuo*, acidified to pH 2 with sulphuric acid and subjected to the same five-funnel extraction procedure with phenol as is described above. The first eight phenol charges were pooled and evaporated as above to give fractions *PVP* and *PWP* respectively, which contained more than 90% of the N present in fractions *PV* and *PW*. The aqueous residues *PVR* and *PWR* were discarded.

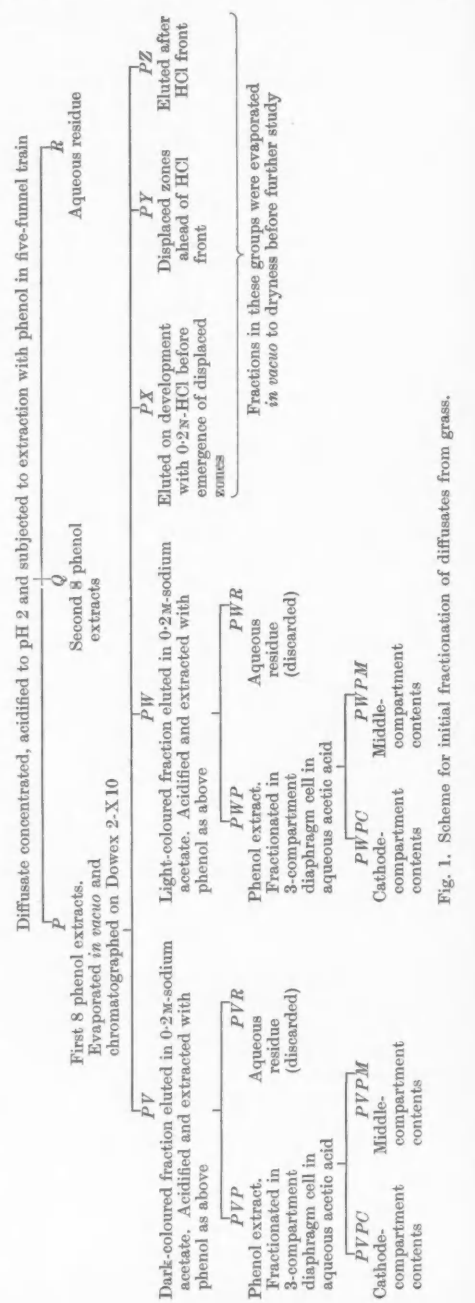
Diffusate concentrated, acidified to pH 2 and subjected to extraction with phenol in five-funnel train
P
Q
R
First 8 phenol extracts. Evaporated in vacuo and chromatographed on Dowex 2-X10
Second 8 phenol extracts
Aqueous residue
PV
PW
PX
PY
PZ
Dark-coloured fraction eluted in 0·2M-sodium acetate. Acidified and extracted with phenol as above
Light-coloured fraction eluted in 0·2M-sodium acetate. Acidified and extracted with phenol as above
Eluted on development with 0·2N-HCl before emergence of displaced zones
Displaced zones ahead of HCl front
Eluted after HCl front
PVP
PVR
PWP
PWR
Phenol extract. Fractionated in 3-compartment diaphragm cell in aqueous acetic acid
Aqueous residue (discarded)
Phenol extract. Fractionated in 3-compartment diaphragm cell in aqueous acetic acid
Aqueous residue (discarded)
Fractions in these groups were evaporated in vacuo to dryness before further study
PVPC
PVPM
PWPC
PWPM
Cathode-compartment contents
Middle-compartment contents
Cathode-compartment contents
Middle-compartment contents

Fig. 1. Scheme for initial fractionation of diffusates from grass.

PVP and *PWP* were then fractionated to separate materials possessing basic ionizing groups from neutral and acidic materials. This was done by the three-compartment cell procedure of Synge (1951). The material, dissolved in 5% (v/v) aqueous acetic acid, was placed in the middle compartment. The contents of the cathode compartment were removed approx. 7, 24 and 48 hr. after starting the run, being replaced with fresh 1% (w/v) aqueous NH_3. This was to decrease the time during which material migrating to the cathode was exposed to alkaline conditions and to the possibility of undergoing reaction at the electrode. The removed catholytes were evaporated to dryness and the residues weighed before pooling them. The greatest part of the material had migrated in the first 7 hr. of the run; during the last 24 hr. only about 2% of the total material migrating arrived in the catholyte; this could well have been neutral material carried from the middle compartment by diffusion and electroendosmosis. The pooled catholytes (*PVPC* and *PWPC*) and the final middle-compartment contents (*PVPM* and *PWPM*) were evaporated to dryness and stored at 0° for further study.

Preparative zone electrophoresis in silica jelly with a volatile buffer

In recent years volatile buffer solutions (especially of pyridine and acetic acid) have found increasing use in preparative zone electrophoresis. The inorganic nature of silica jelly gives it a number of advantages as supporting material in organic and biochemical work. However, a disadvantage of the usual procedures for making silica jelly from water glass has been that if the otherwise desirable enclosed-jelly procedure *B* of Consden, Gordon & Martin (1946) is used in the electrophoresis non-volatile buffer components are necessarily present. We are grateful to Dr Helge Laurell for suggesting to us the use of methyl silicate as a water-miscible source of silicic acid with a convenient gelling time (Ingelman & Jullander, 1945; cf. Ingelman & Laurell, 1947). We have found that with pyridine–acetic acid buffer at about pH 6 this gives very useful jellies. With buffers above this pH a curdy precipitate forms while at lower pH values gelling is excessively delayed. For our particular purpose—the fractionation of organic acids—this limitation has not been a serious one. It may well turn out that jellies made from silicic acid sols prepared from sodium silicate by ion exchange are more versatile. It is of interest that electroendosmosis (determined by fructose or *N*-2:4-dinitrophenylethanolamine markers) was towards the anode, which is opposite to that found in silica jelly with inorganic buffers. We attribute this reversal to the adsorption of pyridinium ions on the gel structure, conferring a positive charge.

On freeze-drying, the silica jelly gives a fine friable powder which is very conveniently handled for extraction in chromatogram tubes by a suitable solvent.

In the present experiments we used as the gelling solution aqueous (v/v) 5% A.R. pyridine; 3% A.R. acetic acid; 4% methyl silicate (prepared according to Voronkov & Dolgov, 1951). The methyl silicate was added with good mixing immediately before pouring the jelly. The trough described by Ellfolk & Synge (1955) or a broader one of the same length and depth was used. After 2–3 hr. the trench for inlaying was cut near the cathode end of the jelly. Electrodes were of Pt wire and were perfused with the above buffer (without methyl silicate). The inlaying solution contained up to 50 mg. of the material to be fractionated/ml., and was otherwise the same as the gelling solution except that for every acid equivalent of the material to be fractionated an equivalent of acetic acid was omitted. When the inlay had gelled, the electrophoresis was run at 2·3 v/cm., which gave a current density of about 23 mA/cm.^{2}. The rather low potential gradient was necessitated by the high ionic strength of the buffer; however, as the buffer components often had an appreciable solvent effect on the materials being fractionated, we did not wish to decrease their concentration. At the end of the run filter-paper prints were taken, dried at 105° in the oven and examined for fluorescence and non-volatile acids and by the reaction of Rydon & Smith (see below). The jelly was then cut into fractions as desired, which were freeze-dried in beakers the tops of which were covered with filter paper, to prevent blowing away of the dried silica. The resulting friable powder was packed into chromatogram tubes and extracted successively with three column's lengths of the phenol-rich and phenol-poor phases of the system phenol (freshly distilled A.R.)–water. The combined extracts from each fraction were evaporated to dryness *in vacuo* with repeated addition of water.

Partition chromatography on kieselguhr

This has been done with Hyflo Super-Cel (Johns-Manville Co., London) as support; with many of the more complicated fractions investigated, paper chromatography showed that serious tailing and irreversible adsorption occurred on cellulose, which contra-indicated preparative work with cellulose powder. The solvent systems used were phenol–water and butanol–(acetic acid)–water in various proportions. Hyflo Super-Cel treated according to Howard & Martin (1950) was used when it was wished to have the organic phase stationary (cf. Ellfolk & Synge, 1955; Bettelheim, 1956). Stationary phase was mixed with kieselguhr (2 ml./3 g.) and the mixture packed as a slurry in moving phase. Temperature was maintained without special precautions in the range 12–18° with a variation of not more than 3° during each run. Fractions when re-chromatographed in the same system gave satisfactorily narrow zones.

Other analytical procedures

General. Evaporation was done below 40° *in vacuo* unless otherwise stated. Grass samples were dried to constant weight in an oven at 105°. Otherwise dry matter was determined by drying to constant weight in a vacuum desiccator over H_2SO_4 and soda lime. Optical rotation was read to ±0·01° in a 0·5 dm. tube at 18–23°. Fluorescence was observed under an Osram 45 MBW/V ultraviolet lamp (G.E.C. Ltd.). Mol.wt. by f.p. depression was determined as described by Findlay (1933) with a Beckmann thermometer.

Elementary analyses. For N, grass samples were analysed fresh by a macro-Kjeldahl procedure, other materials by a micro-Kjeldahl procedure. For both, a Cu–Se catalyst was used. C, H, etc. were determined by F. and E. Pascher, Bonn.

Acid equivalent weight. This was determined by titration with 0·01N-$Ba(OH)_2$, bromothymol blue being used as indicator. Titrated specimens were often further analysed for N, as the reagents gave a low blank in the Kjeldahl determination.

Acid hydrolysis. Unless otherwise stated this was in 6N-HCl for 24 hr. in sealed evacuated tubes at 105°. At the end of the hydrolysis the mixtures were usually evaporated to dryness to remove excess of HCl.

Carboxyl N. This was determined by the ninhydrin–CO_2 procedure as modified by Synge (1951) at pH 4·7 on material before and after acid hydrolysis as above. However, no appreciable evolution of CO_2 was noted with unhydrolysed samples in the absence of ninhydrin with any fractions obtained from grass diffusates by extraction into phenol. Accordingly, with these the blank determination in absence of ninhydrin was omitted and a simple reagent blank was used for correcting results with both hydrolysed and unhydrolysed samples.

Ammonia in evaporated hydrolysates. This was determined according to Conway & O'Malley (1942), the NH_3 being liberated with K_2CO_3.

Filter-paper ionophoresis. This was done by the procedure of Kunkel & Tiselius (1951) as adapted by Ellfolk & Synge (1955), with a variety of volatile and non-volatile buffers.

Filter-paper chromatography of amino acids. This was done by the two-dimensional procedure described by Synge (1951). Treatment of the specimens with H_2O_2 was usually omitted, as sulphur-containing amino acids were, at most, minor components of the fractions studied. With simpler mixtures of amino acids or where the available specimen was small, one-dimensional filter-paper chromatography with butanol–acetic acid–water (8:1:10, by vol.) was sometimes used.

Spray reagents. For free amino acids, etc., ninhydrin was applied in the usual way. For BAA, etc., the Cl_2–starch–KI procedure of Rydon & Smith (1952) was found very useful and sensitive. Particularly when papers had been moistened with organic solvents, it was advantageous to dry them at 105° in the oven for 5–10 min. before placing them in Cl_2 gas. Non-volatile acids were detected by spraying with bromocresol green (Ranson, 1955).

Carbohydrates. Reducing sugar was determined (on samples previously adjusted to neutral pH) by the iodimetric procedure of Somogyi (1945), by using reagent to which iodate had previously been added. Total carbohydrate was determined by an orcinol–H_2SO_4 method, with a reaction time long enough to minimize differences between different kinds of sugar residues (Sørensen & Haugaard, 1933; Pirie, 1936; Vasseur, 1948; Brückner, 1955). The sample (containing 10–100 μg. of carbohydrate) in 0·2 ml. of water was mixed with 2 ml. of 0·2 % (w/v) orcinol (British Drug Houses Ltd.) freshly dissolved in 66% (v/v) cold aqueous H_2SO_4. The mixture was heated for 12 min. at 100° and cooled. Its absorption was compared with that of a blank reaction mixture in an EEL portable colorimeter with a blue 303 filter (Evans Electroselenium Ltd., Harlow, Essex). Results were read from the linear calibration curve obtained with known amounts of glucose treated in the same way.

RESULTS

Initial fractionation

Table 2 gives an over-all picture of the results of applying the scheme of fractionation set out in Fig. 1 to three recent batches of grass. The fractions obtained seem to be reasonably reproducible both in amount and nature. Some of the more detailed chemical information obtained on the fractions is set out below. These studies sometimes relate to material isolated by somewhat different procedures before the present scheme had been worked out. The fractions studied can, however, fairly readily be correlated with those of the present scheme. Fractions are related, throughout this paper, to the total N of the original diffusate or to the dry matter of the original grass after allowance is made for any samples removed for analysis etc. at each stage of the fractionation.

Glycosides in the phenol extracts and their relation to BAA

Diffusate from grass specimen II, when subjected to the fractionation procedure of Synge (1951) with successively four- and three-compartment cells, gave in fraction 3-*M* 5·4 % of its N (carboxyl N before hydrolysis, 13 % of total N, rising to 59 % after hydrolysis). When this same diffusate was subjected, without preliminary acidification, to phenol extraction as above and the material extracted by the first eight charges of phenol was then fractionated by the four- and three-compartment cell procedure, the corresponding fraction (II-*PM*) contained 5·2 % of the N of the diffusate (N, 0·63 % of dry matter; carboxyl N before hydrolysis, 6 % of total N rising to 48 % after hydrolysis). The aqueous residue from the phenol extraction when subjected to the four- and three-compartment cell procedure gave negligible BAA in the corresponding fraction.

Thus fraction II-*PM* evidently contains the same BAA as the fractions 3-*M* of Synge (1951). It may be assumed to correspond to the aggregate of fractions *PVPM*, *PWPM*, *PX*, *PY* and *PZ* of the scheme in Fig. 1. The content of BAA in II-*PM* was very similar to that in this aggregate of fractions (Table 2), although the latter were associated with less dry matter, which was presumably fixed on the ion-exchange resin during the fractionation.

When fraction II-*PM* was heated in N-H_2SO_4 at 100°, the liberation of reducing sugar appeared to be complete after 2 hr. and amounted (as glucose) to 35 % of the dry matter of II-*PM*. During the time of heating in acid, the solution, which had initially been clear, deposited a brown oily layer. This material partly dissolved on extracting the hydrolysate thrice with equal volumes of ether; the ether extracts on evaporation yielded 31 % of the dry matter of II-*PM* and 8 % of its N, in the form of a yellow, partly crystalline gum with a fragrant odour reminiscent of fresh grass and of eucalyptus oil. A tarry gum remained insoluble (7 % of dry matter of II-*PM*), but could be dissolved in ethanol. It contained 9 % of the N of II-*PM*. The aqueous residue, poured off the tarry gum, yielded

Table 2. *Results of initial fractionation for grass specimens* VI, VII *and* VIII

See text for details of fractions *QV*, *QW*, *QX*, *QY* and *QZ*.

Fraction (Fig. 1)	N of fraction as percentage of N of diffusate			Dry matter of fraction as percentage of dry matter of original grass			N of fraction as percentage of dry matter of fraction			Carboxyl N of fraction as percentage of N of fraction					
										Before acid hydrolysis			After acid hydrolysis		
	VI	VII	VIII	VI	VII	VIII	VI	VII	VIII	VI	VII	VIII	VI	VII	VIII
Unfractionated diffusate	100	100	100	31·6	26·7	23·2	1·6	1·3	1·4	40	—	—	75	—	—
P	16·4	22·8	22·8	2·5	3·1	2·3	3·4	3·0	3·3	25	19	27	53	53	43
Q	6·7	7·7	8·8	1·0	1·1	1·8	3·4	2·5	1·6	35	36	44	68	62	64
R (by diff.)	76·9	69·5	68·4	28·1	22·5	19·1	—	—	—	—	—	—	—	—	—
PV	8·9	12·2	11·2	—	—	—	—	—	—	—	—	—	—	—	—
PW	2·7	5·8	5·2	—	—	—	—	—	—	—	—	—	—	—	—
PX	1·4	1·8	0·7	0·11	0·18	—	6·6	3·4	—	—	—	24	—	45	51
PY	0·96	1·2	0·6	0·17	0·18	—	3·0	2·3	—	3	3	—	95	88	81
PZ	0·4	0·88	0·6	0·06	0·13	—	3·3	2·3	—	22	18	—	63	74	78
PVP	8·1	10·6	—	0·63	0·63	—	6·5	5·8	—	15	13	—	36	34	—
PVR (by diff.)	0·8	1·6	—	—	—	—	—	—	—	—	—	—	—	—	—
PWP	2·5	5·8	—	0·13	0·22	—	9·7	9·1	—	5	12	—	41	38	—
PWR (by diff.)	0·2	0	—	—	—	—	—	—	—	—	—	—	—	—	—
PVPC	5·4	8·9	—	0·26	0·26	—	10·4	11·9	—	22	24	—	26	25	—
PVPM	2·7	1·7	—	0·32	0·28	—	4·3	2·1	—	6	6	—	41	38	—
PWPC	1·5	3·9	—	0·050	0·085	—	14·0	16·0	—	14	15	—	55	42	—
PWPM	1·0	1·4	—	0·086	0·105	—	5·9	4·6	—	0	3	—	21	16	—
QV	2·75	2·3	—	—	—	—	—	—	—	—	—	—	—	—	—
QW	1·57	2·6	—	—	—	—	—	—	—	—	—	—	—	—	—
QX	0·31	0·37	—	0·029	0·025	—	5·6	5·1	—	19	—	—	53	44	—
QY	0·51	0·66	—	0·26	0·27	—	1·0	0·85	—	0	0	—	92	30	—
QZ	0·17	0·29	—	0·033	0·063	—	2·7	1·6	—	24	—	—	64	37	—

15 % of the N of II-*PM* as NH_3 and 9 % as carboxyl N (rising to 56 % after complete acid hydrolysis). On qualitative filter-paper chromatography in ethyl acetate–acetic acid–water (Jermyn & Isherwood, 1949) and on filter-paper electrophoresis in borate–phosphate buffer at pH 7 (Consden & Stanier, 1952) the aqueous residue gave carbohydrate spots agreeing in behaviour with that of a mixture of glucose with smaller amounts of galactose and rhamnose.

This experiment suggested that by hydrolysis with N-H_2SO_4 for 2 hr. at 100° considerable glycosidic material could be removed from II-*PM* as water-insoluble aglycone and free sugar without liberating much of the BAA. Water–phenol partition chromatography of II-*PM* on dichlorodimethylsilane-treated kieselguhr showed a complicated distribution of BAA. That obtained on chromatographing the aqueous hydrolysis residue (after removal of sulphate with baryta) was not substantially different, though the amount of N in the fast-moving new peak containing free sugars had increased somewhat over that in the corresponding fraction from unhydrolysed material (Fig. 2). This, with the apparently complete liberation of amide N under the conditions of hydrolysing the glycosides, led us in later work to abandon the use of acid hydrolysis and to investigate other milder methods for fractionating the BAA. As mentioned above, these products could be effectively fractionated by partition chromatography with various solvent systems, and gave a multitude of fractions, some crystalline, each of which yielded on hydrolysis nearly all the different amino acids present before fractionation (cf. Synge & Wood, 1954). Fraction II-*PM* and most derived fractions before acid hydrolysis gave purple or green colours with $FeCl_3$ and their solutions turned strong yellow in aqueous alkali or under the conditions of the Van Slyke amino-N determination with nitrous acid, which we took to indicate the presence of phenolic and polyphenolic residues.

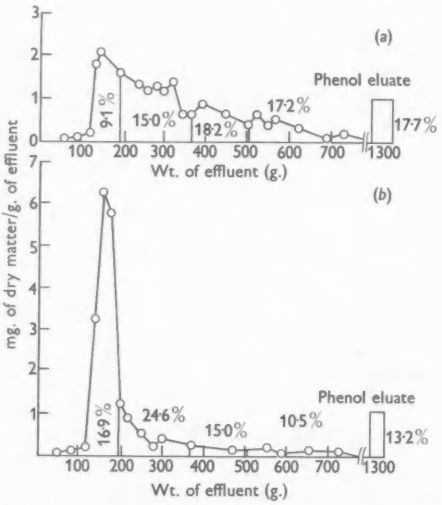

Fig. 2. Water–phenol chromatograms on dichlorodimethylsilane-treated kieselguhr (87 g.) (phenol phase stationary). Columns 10·5 cm. × 5·4 cm.: (*a*) II-*PM*, 560 mg. dry matter; (*b*) aqueous residue from same quantity of II-*PM* after mild acid hydrolysis (see text). After development, the chromatograms were eluted with more than a column's length of stationary phase. The areas marked 'phenol eluate' indicate the dry matter resulting from evaporation of these fractions. The percentages marked on the graph are for carboxyl N after acid hydrolysis for successive grouped fractions; they are percentages of carboxyl N in the material taken for chromatography.

Ion-exchange chromatography of material in the phenol extracts: preliminary studies

Our most detailed studies have been made with a fraction of diffusate from specimen V, obtained by phenol extraction (first eight charges) after acidification to pH 2, followed by fractionation in the three-compartment cell. The resulting middle-compartment contents (referred to as V-*PM*) contained 7·0 % of the N of the diffusate (N, 1·75 % of dry matter; acid equiv.wt., 390; carboxyl N before hydrolysis 7 % of N of V-*PM* rising to 43 % after hydrolysis). This fraction may be assumed to correspond to the same aggregate of fractions in the scheme of Fig. 1 as suggested above for fraction II-*PM*.

Comparison of ion-exchange resins having differing degrees of cross-linkage. Samples of V-*PM* each containing 4 mg. of total N were neutralized with NaOH and fractionated on single columns (1 cm. × 6·7 cm.) of Dowex 2-X10 and of Dowex 2-X2 (200–400 mesh/in., Batch L-2890–35, kindly given by Dr T. S. Work). The fractionation was conducted as described under Methods. Fractions *Z* were eluted in 35–40 ml. of HCl. The results are shown in Table 3. It is seen that, although similar recoveries were obtained from the two resins, 2-X10 gave much more of the N in fractions *V* and *W* than did 2-X2. Moreover, this was associated with the bulk of the brown pigment, which, as noted above, on 2-X10 readily passes into fractions *V* and *W*. On 2-X2 it is strongly held at the top of the column, and even a final elution with 10N-HCl did not liberate much of it. As 2-X10 appeared to give a sharper fractionation and, particularly, to give in fraction *Y* less coloured material, less humin on acid hydrolysis and a higher proportion of the N

Table 3. *Fractionation of* V-*PM on anion-exchange resins of differing cross-linkage*

Fraction	Dowex 2-X10			Dowex 2-X2		
	Percentage of N put on column	N of fraction as percentage of dry matter of fraction	Carboxyl N after hydrolysis as percentage of N of fraction	Percentage of N put on column	N of fraction as percentage of dry matter of fraction	Carboxyl N after hydrolysis as percentage of N of fraction
V + *W*	48·1	—	26	18·8	—	24
X	12·3	—	41	28·5	3·1	11
Y	14·8	1·4	81	27·1	1·7	69
Z	7·1	2·9	49	12·5	2·5	39
Total recovery	82·3			86·9		

Table 4. *Ionophoresis on silica jelly of fractions PVPM and PWPM*

For details see text.

Fraction studied	BAA recovered as percentage of that taken for ionophoresis: Fraction (1) (neutral-migrating with fructose)	Fraction (2) (anionic)	Distance of cut between fractions (1) and (2) to anode side of inlay (cm.)
VI-*PVPM*	39	30	3·3
VI-*PWPM*	51	27	8·5*
VII-*PVPM*	34	46	3·8
VII-*PWPM*	26	68	4·0

* This cut was displaced excessively towards the anode on account of skew running of the fructose zone; this presumably made the values for fractions (1) and (2) too high and too low respectively.

as BAA (displaced by the HCl front), subsequent work has been done with 2-X10.

Demonstration of anionic character of materials not retained by Dowex 2-*X* 10 *in* 0·2 M-*sodium acetate.* Fractions *PVPM* and *PWPM* from specimens VI and VII (Table 2) were submitted to silica-jelly ionophoresis as described above. Approx. 100 mg. of each fraction was mixed with 50 mg. of D-fructose and subjected to ionophoresis in a trough 4 cm. broad for 18 hr. At the end of the run the fructose zone was located by spraying the print with aniline phthalate (Hough, Jones & Wadman, 1950), and the jelly was then cut to give: (1) inlay and fructose zone; (2) all material on anode side of fructose zone. The fractions were worked up and hydrolysed as usual. Carboxyl N was determined on the hydrolysates. Results are shown in Table 4. The values for BAA in fractions (2) are minimal for anionic material in the fractions subjected to ionophoresis since, besides mechanical losses in transfer and taking the print, there may have been incomplete elution of BAA from the dried gel by phenol–water phases (elution was certainly incomplete for the brown pigments present), as well as adsorptive retardation of ions by the gel during migration, leading to their appearance in fraction (1) rather than in fraction (2). The bulk of the brown pigments were seen to migrate faster towards the anode than did fructose, and mostly appeared in fraction (2).

These experiments demonstrate that a high proportion of the BAA which is not retained by Dowex 2-X10 in 0·2 M-sodium acetate (fractions *V* and *W*) is nevertheless anionic in aqueous solution at pH 6.

Detailed results of displacement chromatography of V-*PM′ on Dowex* 2-*X* 10

A somewhat later preparation, V-*PM′*, less efficiently freed from cations in the three-compartment cell, was studied. This had N 3·2 % of dry matter (carboxyl N before hydrolysis, 20 % of total N, rising to 56 % after hydrolysis). The chromatography was done on 18·2 g. of this material as described under Methods. The fractions eluted in sodium acetate (V-*PM′V* and V-*PM′W*) contained 40·0 and 27·9 % respectively of the N put on the column. On further fractionation according to the scheme in Fig. 1 they gave similar results to those with corresponding fractions given in Table 2 and in the preceding paragraph; the brown anionic fractions always showed glycine, alanine, valine, leucine or isoleucine (or both), and glutamic and aspartic acids on hydrolysis, with smaller and more variable quantities of phenylalanine, tyrosine, proline and γ-aminobutyric acid.

The course of the development with HCl is illustrated in Fig. 3, which shows some analytical results on groups of the eluted and displaced fractions (which were pooled largely on the basis of the form of the dry-matter elution curve).

Fractions preceding the displacement zones (V-*PM′X*) (Fig. 3 up to 5·35 l. of effluent). These fractions in aggregate accounted for 7·9 % of the N taken for chromatography. The effluent between 2·85 and 3·4 l. deposited water-insoluble crystals (α) on standing, which increased on concentrating the pooled fractions and, after filtering off, washing with water and drying, amounted to 25 % of the dry matter of the fractions. Similar crystalline material

was isolated in the same way from some, but not all, other batches of grass. The crystals shrank and discoloured but did not melt on heating to above 300° (Found: C, 39·5; H, 3·0; N, 34·6; combustion residue, 0·9 %). The material gave a white fluorescence in ultraviolet light. It dissolved in aqueous NaOH, being reprecipitated on acidification with acetic acid. The murexide test for uric acid (Cole, 1933) was negative, giving a pale yellow with NH_3, which turned somewhat orange on adding NaOH. In 6N-HCl at 105°, α dissolved in the course of 45 min.; after 24 hr. the hydrolysate was pale yellow but without precipitated humin, nor was CO_2 produced during this treatment. Carboxyl N after hydrolysis was 45 % of total N; two-dimensional chromatography for amino acids revealed

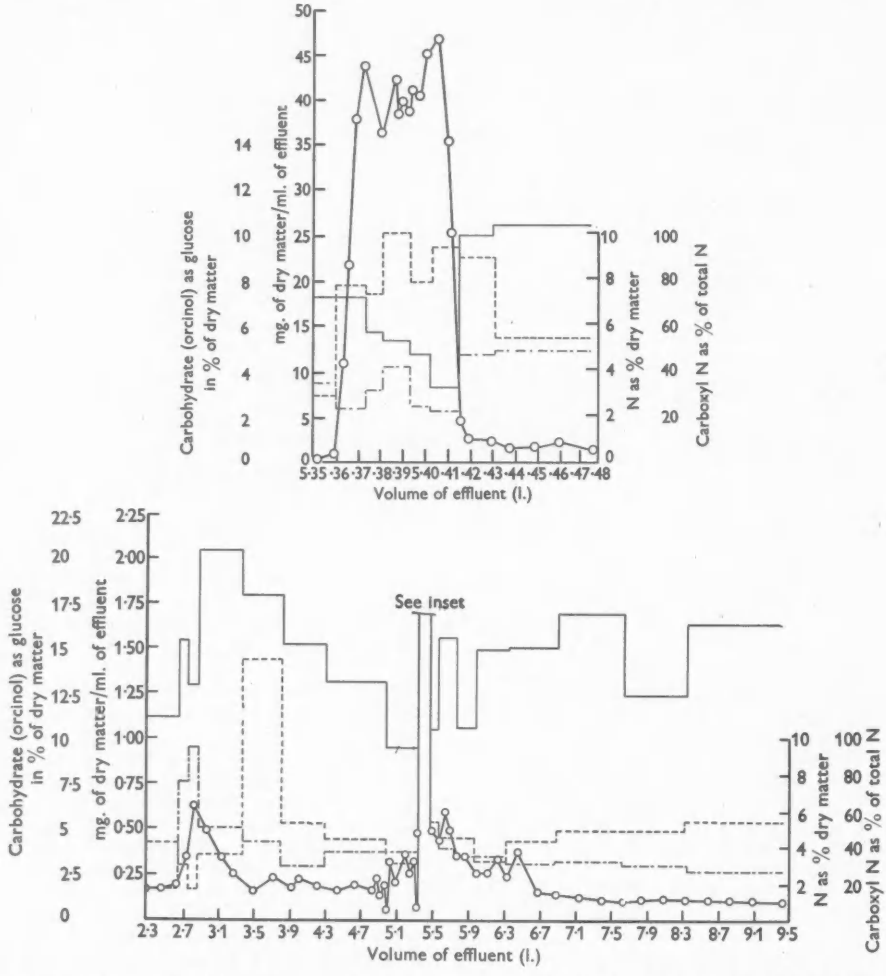
Carbohydrate (orcinol) as glucose in % of dry matter
mg. of dry matter/ml. of effluent
N as % dry matter
Carboxyl N as % of total N
Volume of effluent (l.)
See inset

Fig. 3. Chromatogram of V-*PM'* on Dowex 2-X10 from commencement of development with 0·2N-HCl (see Methods). The inset shows the displacement zones near the break-through of chloride, on different scales. Concentration of dry matter in effluent, O—O. The histograms show analytical results for groups of successive fractions as pooled for analysis: carbohydrate, ——; N as percentage of dry matter, — · —; carboxyl N after acid hydrolysis as percentage of total N, – – –.

only glycine. The properties of α seem not inconsistent with its being a pteridine derivative, but we have not further investigated its nature.

Qualitative examination of hydrolysates of the grouped fractions for amino acids showed glycine, alanine, valine, leucine or isoleucine (or both), and glutamic and aspartic acids. Glycine was particularly prominent just before the peak at 2·85 l. γ-Aminobutyric acid and β-alanine were detected in the region 2·9–4·3 l.

The high N content, low proportion of carboxyl N on hydrolysis, high carbohydrate content and predominance of glycine in hydrolysates of fractions close to the peak at 2·85 l. suggest the presence of nucleosides etc. in this part of the chromatogram (cf. Markham & Smith, 1949). These fractions, however, have not been investigated in detail. [*Note added* 7 May 1958: Corresponding fractions (from the peak regions of VII-*PX* and VII-*QX*) dissolved in 10% (v/v) aqueous acetic acid showed a rather flat maximum of ultraviolet absorption at 260–265 mμ ($E^{0.001\%}_{1cm.}$ 0·14 and 0·105 respectively).]

Displacement zones (V-*PM'Y*) (5·35–5·415 *l.*): *further fractionation by electrophoresis.* These fractions in aggregate accounted for 10·5% of the N taken for chromatography. We have studied this fraction in more detail than any other because of the high proportion of its N represented by BAA and because the acids composing it, being retained on the resin, are probably chemically simpler than those in *V* and *W* fractions, which do not seem to be able to enter the resin. Moreover, the substances in this group are of low carbohydrate content (see Fig. 3) and give little humin on acid hydrolysis.

Samples from the different fractions were subjected to filter-paper ionophoresis in pyridine–acetic acid buffer at pH 6 and examined for fluorescence, non-volatile acid and for material staining with Cl_2–starch–KI. The results are shown in Fig. 4. On the basis of these tests, the fractions were pooled into five groups, as shown in Figs. 3

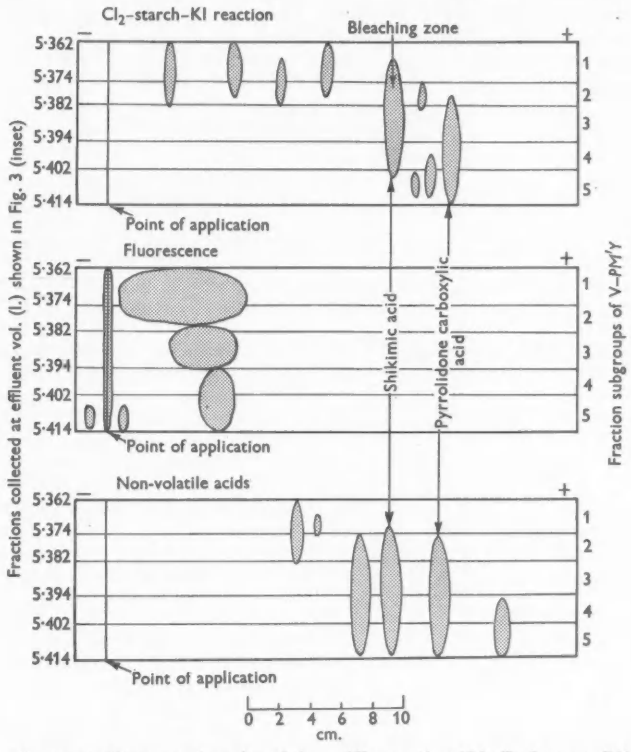

Fig. 4. Distribution of zones on filter-paper ionophoresis in pyridine–acetic acid buffer [aqueous 7% (v/v) pyridine–1% (v/v) acetic acid; pH approx. 6] of fractions (V-*PM'Y*) displaced by HCl (Fig. 3, inset). Ionophoresis was for 6 hr. at 7·5 v/cm.

(inset) and 4 (V-*PM′Y* 1, 2, 3, 4, 5). Each of these was then subjected to ionophoresis in silica jelly, and the analytical results are plotted in Fig. 5. All the fractions which contained appreciable amounts of carboxyl N showed, after acid hydrolysis, glycine, alanine, valine, leucine or isoleucine (or both), aspartic and glutamic acids. Many, especially from groups 4 and 5, also showed phenylalanine, tyrosine, γ-aminobutyric acid and β-alanine. It was obvious that fraction V-*PM′Y* 3*e*, which gave mainly glutamic acid on hydrolysis, contained chiefly pyrrolidonecarboxylic acid, which we identified chromatographically and ionophoretically with authentic material (cf. Ellfolk & Synge, 1955). The other forms of BAA seemed to fall into two groups, one displaced ahead of and one behind the pyrrolidonecarboxylic acid. The former fractions represented a substantial proportion of the BAA and were of high N content. They also contained material, migrating anionically nearly as fast as pyrrolidonecarboxylic acid and well ahead of fluorescent material, which gave on acid hydrolysis little humin and the typical

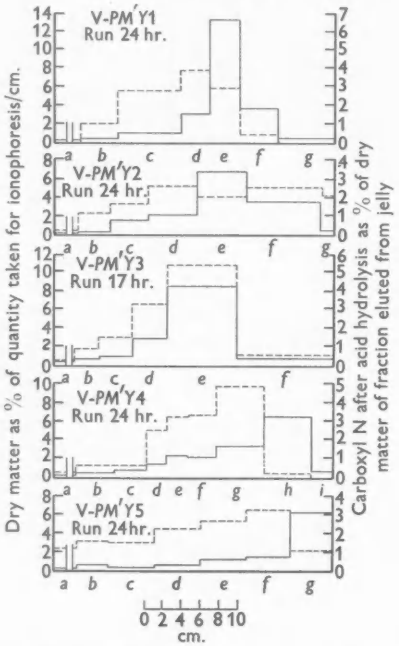

Fig. 5. Distribution of dry matter (——) and carboxyl N (– – –) in fractions obtained after silica-jelly ionophoresis of fraction groups V-*PM′Y* (1–5) (Figs. 3, 4). The fractions taken for further study are indicated by letters along the abscissae.

mixture of amino acids. Accordingly, we decided to investigate such fractions as V-*PM′Y* (1, 2)*e* in greater detail (see below).

Fractions eluted behind hydrochloric acid front (V-*PM′Z*). The successive fractions (Fig. 3) did not show obvious differences in chemical composition. On acid hydrolysis, all gave the same mixture of amino acids, including phenylalanine and tyrosine, as in fraction V-*PM′Y*, while the fractions were more deeply coloured both before and after hydrolysis. They were not further studied.

Detailed study of an individual fraction (β) rich in BAA: occurrence of shikimic acid

Fractions V-*PM′Y* 1*e* and V-*PM′Y* 2*e* were selected for reasons given above and pooled. On partition chromatography (phenol–water) we recovered the bulk both of BAA and dry matter in a relatively sharp peak (R 0·73, corresponding to a partition coefficient of 3·5 in favour of the stationary aqueous phase). This low solubility in phenol proved useful for freeing the material to be studied from pyrrolidonecarboxylic acid, which runs fast on such columns (cf. Ellfolk & Synge, 1955). Pyrrolidonecarboxylic acid had a similar ionophoretic mobility and occurred in the displacement zone immediately following our material; it was therefore a major contaminant of the fractions being studied. However, the low solubility in phenol also meant that poor total yields of this fraction had been extracted from the original diffusate. Accordingly, in the preparations from specimens VI and VII we took off 16 charges of phenol phase from the extraction train and pooled and worked up the second 8 charges separately (fractions Q, Fig. 1 and Table 2). The appropriate displacement zones (subfractions of PY and QY) were subjected to silica-jelly ionophoresis. In each case the prints from the electrophoresis showed, with Cl_2–starch–KI, a zone migrating anionically at about six-sevenths of the velocity of the well-staining sharp pyrrolidonecarboxylic acid zone, which instead of staining caused bleaching of the faint background stain on the paper. This zone was found, nevertheless, to be rich in BAA, and was cut out and worked up. The combined 'bleaching zones' from all the electrophoreses were subjected to phenol–water chromatography, with the results shown in Fig. 6. The fractions of the main peak were pooled. Amounts and properties of the resulting fractions β from specimens V, VI and VII are given in Table 5. Fractions β were colourless and readily soluble in water. They gave little or no humin on acid hydrolysis. C, H and S were present; halogen, P and combustion residue were absent. [P was kindly determined by Mr W. Duncan by the method of Berenblum & Chain (1938) after washing in H_2SO_4.]

Hydrolysis of fractions β in 6N-HCl at 105° for 24 hr. led to the formation of much crystalline N-free acidic material poorly soluble in water and readily extracted into ether, for which elementary analysis suggested an aromatic composition. Hydrolysis in N-HCl or H_2SO_4 for 6 hr. at 100° gave rise to little of this material, while nevertheless liberating most of the amino acids. No CO_2 or volatile acid was liberated. This tendency to aromatization on heating in concentrated acid, the high negative optical rotation and the bleaching in the Cl_2–starch–KI reaction suggested that shikimic acid might be present in our fractions. Richardson & Hulme (1955) found shikimic acid in *Lolium perenne* in unstated yield and (1957) in lucerne. As in our work, it preceded pyrrolidonecarboxylic acid among the zones of plant acids displaced from Dowex 2 by HCl (cf. Hulme & Richardson, 1954; Davies & Hughes, 1954). The electrophoretic mobility of β is what would be expected for a monobasic acid of this molecular weight. An authentic specimen of shikimic acid, kindly given by Dr D. E. Hathway, was found, on paper chromatography in phenol–water and in butanol–acetic acid–water (cf. Hulme & Richardson, 1954; Richardson & Hulme, 1957), to migrate identically with our 'bleaching' fraction and to bleach the background in the Cl_2–starch–KI reaction. An authentic specimen of dihydroshikimic acid, also given by Dr Hathway, migrated at similar rates but did not show the bleaching reaction. Its presence in fractions β is by no means excluded (cf. Hathway, 1956).

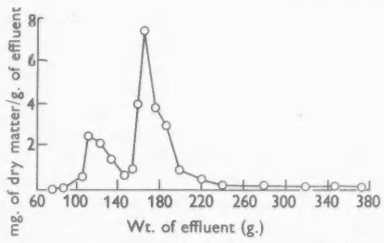

Fig. 6. Phenol–water partition chromatogram of 'bleaching zone' material from VII-*PY* and VII-*QY* (266 mg.) (see Text). Column, 13·3 cm. × 3·6 cm. (41 g. of kieselguhr). The main peak between 143 and 241 g. of effluent was collected as fraction VII-β.

Table 5. *Yields and properties of fractions* β

	V-β	VI-β	VII-β
Yield (N as percentage N of original diffusate)	—	0·028	0·09
Yield (dry matter as percentage dry matter of original grass)	—	0·0076	0·019
$[\alpha]_D$ in water (c, 2)	−155°	−152°	−121°
Acid equiv.wt.	197	182	207
N as percentage dry matter of fraction	3·2	1·9	1·7
Mol.wt. by f.p. depression in water	193	—	—
Carbohydrate content (percentage as glucose by orcinol method)	5	5	—
After hydrolysis in 6N-HCl 24 hr. at 105°	N as percentage total N of fraction		
Carboxyl N	63	61	87
Ammonia (Conway)	24	19	9·4
Ammonia*	20·8	12·5	
Aspartic acid*	6·8	7·6	
Glutamic acid*	13·6	6·6	
Glycine*	13·7	6·8	
Alanine*	10·7	5·2	
Valine*	10·9	4·1	
Methionine*	0·25	0·18†	‡
Leucine*	4·1	0·27	
Threonine*	0	0·85§	
Serine*	0	3·0§	
Isoleucine*	0	0·56	
Phenylalanine*	0	0·50	
Lysine*	0	0·46	
Unknown*‖	1·3	0	

* Determined in the laboratory of Drs S. Moore and W. H. Stein by the automatic procedure of Spackman, Stein & Moore (1956).

† As sulphone on column.

‡ Presence of glutamic and aspartic acids, glycine, alanine, valine, leucine or isoleucine (or both) demonstrated qualitatively by filter-paper chromatography.

§ Uncorrected for destruction in hydrolysis.

‖ Position on chromatogram consistent with γ-aminobutyric acid; calc. with extinction value for leucine.

Further fractionation of β: presence of bound oxalic acid

The evidence that fractions β consisted mainly of shikimic acid accompanied by a complicated and variable mixture of BAA led us to undertake further fractionation. Partition chromatography in butanol–acetic acid–water, at first on paper and later on kieselguhr, gave separation of BAA from shikimic acid and some further indication of the complexity of the mixture of compounds incorporating BAA. Fig. 7 shows the chromatographic fractionation of VII-β and Table 6 some properties of the resulting fractions.

No attempt has been made to study in greater detail the BAA still mixed with shikimic acid in the readily crystallizing fractions VII-β3 and VII-β4. VII-β2 obviously corresponded with two closely adjacent Cl_2–starch–KI-positive spots seen on the filter-paper chromatograms and may well have consisted of two or three individual compounds; the small amount prevented further study. VII-β1 slowly crystallized as rosettes on keeping in the laboratory (though it would not crystallize in the desiccator). The crystals were not very soluble in water, but dissolved readily in 50% (v/v) aqueous ethanol. Nevertheless, the material was obviously still a mixture. After 24 hr. hydrolysis in 6N-HCl at 105°, 8% of oxalic acid was found by the method of Halliwell (1950). This is about one-third of that necessary to form *N*-oxalyl bis-amino acid from all the amino acid residues present. Other common plant acids were not detected by two-dimensional filter-paper chromatography (Ranson, 1955, p. 558) in acid hydrolysates of VII-β1. VII-β1 gave no appreciable consumption of periodate or liberation of formic acid under the conditions of Perlin (1954) (less than 0·2 mole/g. equiv.wt. in 24 hr.). This indicated the absence of α-diol groupings, of shikimic acid and of carbohydrate material. Under the same conditions shikimic acid consumed 4·4 mol. prop. of periodate and liberated 1·9 equiv. of acid/mole.

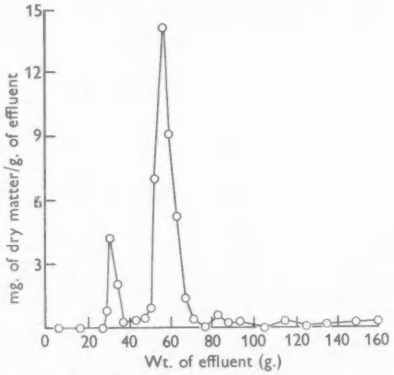

Fig. 7. Further fractionation of VII-β (165 mg.) by partition chromatography in butanol–acetic acid–water (100:12·5:125, by vol.). Column, 13·6 cm. × 2·0 cm. (15 g. of kieselguhr). Fraction VII-β1 comprised effluent from 28 to 40·5 g.; VII-β2, from 40·5 to 48 g.; VII-β3, from 48 to 57·5 g.; VII-β4, from 57·5 to 78 g.

Bound oxalic acid in fraction PY

Oxalic acid was determined according to Halliwell (1950). None was found in VIII-*PY*; after acid hydrolysis, however, this fraction yielded 0·13 mole of oxalic acid/atom of N.

DISCUSSION

Our meagre knowledge of the low-molecular compounds which occur in plants and which yield amino acids on hydrolysis was reviewed by Synge (1955). As concerns higher plants, there is growing up a voluminous but inconclusive literature. Much of the inconclusiveness is due to differences of extraction procedures and to lack of evidence bearing on the molecular weight of the materials studied; also in many papers quantitative information on yields and composition is completely lacking. However, nobody so far seems to have searched by valid methods in plant material and failed to find bound amino acids (BAA) present as

Table 6. *Yields and properties of fractions resulting from partition chromatography of* VII-β *in butanol–acetic acid–water (see Fig. 7)*

Fraction	VII-β1	VII-β2	VII-β3	VII-β4
Yield (percentage of dry matter taken for chromatography)	11·4	2·4	48·5	29·7
$[\alpha]_D$ in water (*c*, 2)	−10°	—	−157°	−138°
Acid equiv.wt.	196	—	207	194
N as percentage dry matter of fraction	8·0	—	2·1	1·1
Amino acids present after hydrolysis (one-dimensional filter-paper chromatography)				
Major	Leu, Val, Ala	Ala	Gly and/or Glu, Asp	Ala
Minor	Gly and/or Glu, Asp	Gly and/or Glu, Asp	—	—

compounds of low molecular weight. Of the better-defined compounds, the following may be added to those previously reviewed: γ-Glutamyl-β-amino-propionitrile has been isolated from sweet-pea seeds (*Lathyrus odoratus*) (McKay, Lalich, Schilling & Strong, 1954; Dasler, 1954; Schilling & Strong, 1954, 1955). Dupuy & Lee (1954) isolated a similarly toxic nitrogenous substance from seeds of *Lathyrus pusillus*. Virtanen & Berg (1954) gave evidence for the occurrence of γ-glutamylalanine in germinating peas. Rinderknecht (1957) isolated γ-glutamyl-*S*-methylcysteine from lima beans and gave evidence for the simultaneous occurrence of the derived sulphoxide and of γ-glutamyl-leucine. Price (1957) found a new thiol compound, possibly a glutathione analogue, in leguminous plants. Eastwood, Hughes, Ritchie & Curtis (1955) studied evolidine, a crystalline substance isolated from *Evodia xanthoxyloides*, and suggested that it is a cyclic heptapeptide. Hassall & Reyle (1955) isolated from the fruit of *Blighia sapida* two toxic substances which they called hypoglycin A and B. Of these, A was crystalline and both seemed to be peptides. Later work suggests that B is a glutamyl derivative of A, which is a free amino acid of novel cyclic structure (Holt & Leppla, 1956; Holt, Leppla, Kroner & Holt, 1956; Wilkinson, 1958). Bieber & Clagett (1956) isolated from young wheat plants, as the *N*-2:4-dinitrophenyl derivative, what appeared to be a hexapeptide having aspartyl-threonyl as its *N*-terminal sequence. Borriss & Schneider (1955) isolated chromatographically a ninhydrin-staining component of an aqueous extract of seeds of *Agrostemma githago*, which gave several amino acids after acid hydrolysis (see also Schneider, 1955). Virtanen & Linko (1955) isolated a monoacetylornithine from *Asplenium nidus*, apparently identical with that obtained by Manske (1937, 1946) from *Corydalis* spp. It is surprising that it has not yet been rigorously determined whether this is the N^{α}- or N^{δ}-acetyl derivative. Andreae & Good (1955) identified indolylacetyl-aspartic acid in pea seedlings. Winterfeld & Leiner (1956) reported further studies on viscotoxin fractions, and now consider that these are composed of amino acid and carbohydrate residues only. A few of the isolations of less well-defined products are mentioned below where relevant to the discussion.

The work which we now report has not led to the isolation or chemical characterization of any definite compound. It has, however, helped to show how complicated can be the mixture of compounds of this class normally present in a green plant.

We have purposely avoided during the fractionation conditions which would encourage reaction between amino acids and sugars, leading to the formation of *N*-glycosides, etc. Such compounds undoubtedly account for some of the 'peptide' reported in extracts, particularly of dried plant material (Synge, 1955). Of course, since free amino acids and reducing sugars are present together in the tissues of the living plant, such reaction products may be naturally present in the starting material. However, compounds of this type are not likely to be readily extractable into phenol, and thus should not bulk large in the fractions described here.

From the present work we can recognize in the diffusates four categories of BAA:

(1) *True peptides.* Such compounds seem to be present in fractions *PWPC*. They are positively charged in aqueous acetic acid and migrate cationically. In the fractionation by Synge (1951) they would have been present in fractions 4-*B* and 3-*C* and escaped attention on account of the large amounts of accompanying free amino acids. The present procedure has led to their concentration because: (*a*) phenol has a special affinity for peptide bonds (for references see below); (*b*) the anion-exchange resin, presumably on account of molecular adsorption on its polystyrene skeleton and of differences in p*K* values of amino groups, somewhat retards peptides and aromatic amino acids relative to the other free amino acids and other potential cations (perhaps betaines or alkaloids), which are not retarded and pass to fractions *PVPC*.

(2) *Compounds eluted from the anion-exchange resin in aqueous acetic acid.* These (fraction groups *PX*, *QX*) would be expected to be weakly acidic compounds having p*K* values substantially higher than that of acetic acid (4·7). The relatively high nitrogen content, the low proportion of carboxyl nitrogen after hydrolysis and the predominance of glycine in the hydrolysates suggest the occurrence of derivatives of purines, pyrimidines, pteridines, etc. in at least some of the fractions (see Fig. 3). Markham & Smith (1949) noted that glycine is formed from purines under the conditions usual for the acid hydrolysis of proteins. The high carbohydrate content suggests that the nitrogen compounds may occur in glycosidic union, although the occasional separation by crystallization of the pteridine-like substance α shows that carbohydrate is absent from some of them. BAA other than glycine were present in all the fractions of this group, and further work is required to establish the nature of the compounds present.

(3) *Compounds displaced from the anion-exchange resin by hydrochloric acid.* Ordinary plant acids were also present in these fractions (groups *PY*, *QY*). However, the greater part of their nitrogen was present as carboxyl nitrogen of BAA, and much of the remainder was present as ammonia in the acid hydrolysates. In fractions V-β and VI-β,

the only ones analysed in detail, the ammonia produced was roughly equimolar with the glutamic and aspartic acids (Table 5). Fluorescent and brown–red pigmented compounds were present in *PY* and *QY*, but a substantial proportion of the BAA present had higher anionic mobility and could be separated as colourless and non-fluorescent material. In general the content of carbohydrate and formation of humin on hydrolysis was low. The nature of these compounds is discussed, with that of (4), below.

(4) *Neutral and acidic compounds not retained by the anion-exchange resin at neutral* pH (fraction groups *PVPM*, *PWPM*, *QV*, *QW*). These fractions contained the bulk of the BAA and of the brown pigment of the grass diffusates, and since they consisted largely of anionic material (Table 4) the most reasonable explanation for their non-retention on Dowex 2-X10 is that the molecular weight is too great to permit the anions to enter the pores of the resin. The material in fractions *PZ* and *QZ* is perhaps intermediate in character between our categories (3) and (4), being sorbed but rather unreadily given up by the resin. Similar material may well account for the nitrogen and pigment held irreversibly by the resin. Failure of larger molecules to penetrate resins, as well as their slow or irreversible adsorption, are well-known phenomena (for some references see Mould & Synge, 1952; Waley, 1957; Chaudhry & Saunders, 1956). Asher (1956) found that molasses pigments were largely excluded from Dowex 50-X4 (sodium form), and thus separated them from sugar. Anderson & Hansen (1955) described the sorption of phenolic compounds by Dowex 1 and 2.

It seems reasonable to regard the BAA in categories (3) and (4) as a complicated mixture of *N*-acyl derivatives of amino acids, among which glycine, alanine, valine, leucine or isoleucine (or both), asparagine and glutamine consistently predominate. If the acyl radicals are polybasic and the acylation is more or less at random, this will give rise to a very complicated series of compounds even with the simplest series, the *N*-oxalyl bis-amino acids, for whose presence we have some evidence from fraction β and from the occurrence of bound oxalic acid in fraction *PY*. The idea that we are dealing with *N*-acyl amino acids is further supported by our failure to observe, on paper chromatography of partial acid hydrolysates of these fractions, any ninhydrin-staining material other than the ordinary amino acids and by our attempts to follow partial acid hydrolysis by the Van Slyke nitrous acid procedure for amino nitrogen. We are not sufficiently sure of the reliability of these determinations in the presence of polyphenolic material, humin, etc. to wish to report them in detail. However, in no case did the amino nitrogen exceed the value for carboxyl nitrogen determined by the ninhydrin–CO_2 procedure on the same partial hydrolysate. Both kinds of observation suggest the absence of true peptide groupings (i.e. two or more amino acid residues linked to one another by peptide bonds).

In connexion with the possible occurrence of *N*-oxalyl amino acids the work of Kminek (1936) on bound oxalic acid in sugar-beet juice is of interest. He demonstrated that oxalic acid could be set free by acid hydrolysis of such juices. Allantoin, which was shown to be a source of oxalic acid on alkaline hydrolysis, could not be a precursor. Kminek isolated oxamic acid, $HO_2C{\cdot}CO{\cdot}NH_2$, in amounts sufficient to account for about 25% of the acid-labile bound oxalic acid. This left open the possibility of other forms of bound oxalic acid being present.

Other *N*-acyl amino acids already found in plants include acetylornithine and indolylacetylaspartic acid (referred to above), as well as folic and pantothenic acids and their various derivatives. Birkinshaw, Raistrick & Smith (1942) found fumaryl-alanine in a fungus; the dibasic acid K isolated by Richardson & Hulme (1957) from lucerne is isomeric with this. Some of the present acyl radicals, especially for category (4) above, may be considerably more complicated. The rather high molecular weight and the phenolic and glycosidic character of the fractions suggest tan acids. In this connexion the claim of Nierenstein (1914, 1915) to have found *N*-galloyl-leucine in oak apples may be relevant. Although we have not conclusively shown that the BAA, phenolic materials, glycosidic materials and pigmented and fluorescent materials are a single class of compound, neither have we succeeded in separating them. The possible connexion between the BAA of this category and lignin precursors also deserves consideration, in view of the presence of nitrogen and perhaps BAA in lignin as usually prepared from forage plants (Bondi & Meyer, 1948; Thomas & Armstrong, 1949; de Man & de Heus, 1950; Meyer & Bondi, 1952).

Mention should be made of some studies of plant extracts which have yielded results similar to our own. Biserte & Scriban (1954) have reviewed their studies of diffusible BAA in barley, malt and wort and found, besides some apparently neutral BAA, acidic compounds yielding on acid hydrolysis the same group of amino acids as found by us, including β-alanine and γ-aminobutyric acid. They gave evidence for the presence of pantothenic acid and the homologous derivative of γ-aminobutyric acid. Similar reports of bound β-alanine and γ-aminobutyric acid are found in the work of Champigny (1955) and Champigny & Lioret (1955). Virtanen & Miettinen (1953) studied an ethanolic extract of

young pea plants, and found a substantial amount of BAA therein which was not retained by a sulphonic-type cation-exchange resin. This resembled our material in the amino acids (including β-alanine and γ-aminobutyric acid) which were set free on acid hydrolysis, in its adsorption behaviour on charcoal, in containing bound carbohydrate, and in giving no colour with ninhydrin. However, their products, unlike ours, failed to be fractionated by ionophoresis in silica jelly at pH 7. Miettinen (1957) described the incorporation of [^{14}C]-L-alanine into this fraction after administration of it to flowering pea plants. Porath isolated fractions from a pea-root exudate by charcoal chromatography (1954) and zone electrophoresis (1956). These gave colours with ninhydrin, and may have been peptides. Ferguson & Terry (1954) found a substantial proportion of the nitrogen of herbage extracts not to be retained on columns of Zeo-Karb 215, and therefore presumably devoid of basic groupings. Such of this as was not nitrate may correspond with our categories (2), (3) and (4) above. Pollard & Sproston (1954) isolated a fraction from maple sap by ethanol extraction followed by precipitation with mercuric nitrate. Acid hydrolysis liberated from this fraction a mixture of amino acids similar to that found by us in categories (3) and (4). Pollard & Sproston considered that these were set free from peptide material, since their fraction on two-dimensional paper chromatography showed a ninhydrin-staining spot which was absent on chromatographing acid hydrolysates of the fraction. This cannot be regarded as conclusive evidence for the peptide nature of the material (cf. Synge & Wood, 1956; Holt *et al.* 1956); the matter is the more difficult to judge because the whole fraction, rather than that part of it responsible for the 'peptide' spot, was subjected to hydrolysis. Material yielding similar mixtures of amino acids on hydrolysis has been extracted from paper by Wynn (1949), Hanes, Hird & Isherwood (1952) and Stroh (1956). Hydrolysates of jute likewise yielded amino acids of this group (Sanyal, 1956).

Some technical aspects of the separations

Pardee (1951) and Grassmann & Deffner (1953) have emphasized the affinity of phenol for the –CO–NH– group (cf. Tavel & Signer, 1956). Extraction into phenol from an aqueous phase has been used with good effect for separating protein material from carbohydrates, nucleic acid etc. in studies of a number of animal and microbial products (Morgan & Partridge, 1941; Westphal, Lüderitz & Bister, 1952; Jones, Larsen, Vardaman & Baisden, 1953; Schuster, Schramm & Zillig, 1956; Kirby, 1956). The method should be especially valuable with plant material containing large quantities of carbohydrate. In the present work, the greater part of the BAA was readily extracted into phenol away from the common carbohydrates. Nor did partition chromatography with the system phenol–water present any difficulties when kieselguhr was used as the support; either phase could be held stationary by using appropriately treated kieselguhr (Ellfolk & Synge, 1955; Bettelheim, 1956) and good fractionations were obtained of materials which streaked badly during paper chromatography in the same solvent system.

In the displacement chromatography on Dowex 2 pyrrolidonecarboxylic acid served as a carrier, driving some components ahead of it and leaving others behind. We therefore did nothing to hinder its formation from glutamine during the prolonged acidification of the diffusate for phenol extraction. The usefulness of such carriers, which can be readily eliminated at a later stage, for separating compounds during displacement chromatography has been demonstrated by Westall (1955) in his study of trace components of human urine, where the common amino acids acted in this way. Such a procedure improves the separations obtainable at an early stage of the analysis, when large quantities of material must be handled, but it is not as refined as elution chromatography. The value of elution chromatography on Dowex 2 for fractionating acyl amino acids and similar compounds has been demonstrated by Stein, Paladini, Hirs & Moore (1954), Tallan, Bella, Stein & Moore (1955) and Tallan, Moore & Stein (1956). Such a procedure might be expected to give good results with our end fractions such as β1, which are still complicated mixtures. The behaviour of Dowex 2 resins with other components of our mixtures is discussed above.

The zone electrophoresis in silica jelly with volatile buffer allowed us to handle 0·5 g. batches of acidic material with recoveries which averaged about 90%. The manipulations are simpler than for other zone-electrophoretic procedures on the same scale and the 'tailing' of zones due to adsorption seemed, for our materials, somewhat less on the silica than on filter paper.

SUMMARY

1. Most of the 'bound amino acids' in protein-free diffusates from Italian ryegrass are readily extracted from aqueous solution into phenol.

2. The material in such phenol extracts has been further fractionated on anion-exchange resins, by zone electrophoresis and by partition chromatography.

3. A small proportion of the bound amino acids seems to be in genuine oligopeptides but the greater part is in acidic compounds. These are a very

complicated mixture in which predominantly glycine, alanine, valine, leucine or isoleucine (or both), asparagine and glutamine seem to occur as *N*-acyl derivatives. The acyl radicals may be polyphenolic and glycosidic, although *N*-oxalyl groups may also occur. Some of the amino acids set free on acid hydrolysis may be artifacts from breakdown of nucleosides, pteridines, etc. β-Alanine and γ-aminobutyric acid are also liberated.

4. Reasons are given for the failure of most of the bound amino acids to migrate as anions in the diaphragm-cell procedure of Synge (1951). This led to the wrong conclusion that they were in neutral compounds devoid of ionizing groups.

5. The course of fractionation on anion-exchange resins was very strongly influenced by the degree of cross-linking of the resin, which suggested that some of the compounds are of rather high molecular weight.

6. A useful procedure is described for zone electrophoresis in a silica jelly made from methyl silicate and a volatile buffer. Fractions are eluted after freeze-drying the jelly.

7. The occurrence of shikimic acid in grass extracts is confirmed.

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The Effect of pH Variations on the Activities of C-Esterase*

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In a recent paper, the occurrence in hog's kidney of a new type of esterase, called C-esterase, was demonstrated, which was characterized by the following distinctive properties (Bergmann, Segal & Rimon, 1957). (1) C-Esterase does not hydrolyse di*iso*propyl phosphorofluoridate. In this respect it differs from the A-esterases. (2) C-Esterase is not inhibited by DFP, a property which distinguishes the new enzyme from the B-esterases. (3) C-Esterase is strongly activated by aromatic mercurials.

The characteristics of the new enzyme raise the problem of the underlying hydrolytic mechanism. For the A- and B-esterases information about the active surface has been derived from studies on the pH dependence of ester hydrolysis (Wilson & Bergmann, 1950; Bergmann & Wurzel, 1954; Bergmann, Segal, Shimoni & Wurzel, 1956; Mounter, Alexander, Tuck & Dien, 1957) and studies of inhibitors (Bergmann & Shimoni, 1952). Investigation of the influence of pH variations on the activities of C-esterase, described in the present paper, reveals a close relationship between C-esterase and other groups of hydrolytic enzymes, but points also to certain characteristic differences which so far have not been explained.

* Part of a Ph.D. thesis submitted by S.R. to the Faculty of Science, The Hebrew University, Jerusalem, Israel.

MATERIALS AND METHODS

Substrate hydrolysis. The derivatives of phenyl acetate used in this investigation and the spectrophotometric method of following the progress of their hydrolysis have been described by Bergmann, Rimon & Segal (1958). Enzymic rates throughout this paper represent the

difference between total and non-enzymic rates. The curves, representing non-enzymic hydrolysis as function of pH, have also been given by Bergmann *et al.* (1958).

Inhibitors and activators. Tetraethyl pyrophosphate (TEPP) was a gift of Dr A. D. F. Toy, Victor Chemical Works, Chicago, Ill., U.S.A. As inhibition by TEPP of the B-esterase in the enzyme preparations is complete within a few minutes, and as this phosphate is hydrolysed rapidly by di*iso*propyl phosphorofluoridatase (DFPase) but does not interact with C-esterase, the original method of elimination of B-esterase was modified by incubating the enzyme mixture at room temp. with 10 mM-TEPP for 5 min. before use.

Sodium *p*-chloromercuribenzoate (Sigma Chemical Co.), in concentrations of 10–50 μM, was used as inhibitor of the DFPase present and as activator of C-esterase. In these concentrations the extinction of CMB, in the spectral range suitable for measuring the hydrolysis of phenyl acetates, is negligible.

Enzyme preparations. The C-esterase was purified from hog's-kidney extract as described previously (Bergmann *et al.* 1957). The final solution, representing the fraction precipitated by 30% ethanol, contained usually 45–60 mg. of protein/ml., as determined by addition of an equal volume of 20% (w/v) trichloroacetic acid. When diluted 1:200, the enzyme E-10, which was used in most of the present experiments, hydrolysed 0·4 μmole of mM-*p*-nitrophenyl acetate/ml./hr. Among eight new enzyme preparations, again three were encountered which lost part of their activity upon dialysis during 48 hr. (to the extent of 40, 46 and 50% respectively), possibly owing to removal of a cofactor. The representative of the latter group of enzyme preparations used mostly in the present studies is designated E-9. It had a protein content of 44 mg./ml. and, under the conditions described above, it gave a hydrolytic rate of 0·35 μmole/ml./hr.

Buffers. The pH range required for the present investigation was covered by the use of the following buffers: pH 5·0–6·5 by 0·1 M-sodium acetate; pH 6·5–8·0 by 0·1 M-sodium phosphate and pH >8·0 by 0·1 M-sodium borate or pyrophosphate.

RESULTS

Properties of purified C-esterase

A linear relationship between enzyme concentration and rate of hydrolysis was established. The pS [−log (concentration of substrate, M)]–activity curves of the three phenyl acetates used are also linear in the range 0·1–1·0 mM (Fig. 1), which is adequate for spectrophotometric measurements of rates. At pH 7·5 the descending order of relative velocities is as follows: *p*-nitrophenyl acetate, phenyl acetate, *p*-methoxyphenyl acetate. The order in this series is thus entirely different from the order reported earlier for true cholinesterase, but is similar to that for imidazole-catalysed hydrolysis (Bergmann *et al.* 1958). Accordingly, we may state that with C-esterase rates are the higher the smaller the electron density at the ethereal oxygen atom of the ester group.

Enzymic rates are linear with time at all pH values, at least for the first 10 min. Therefore the pH dependence of hydrolytic activity can easily be derived from the initial portion of the curves, representing rates as function of time.

Recovery of enzyme activity after exposure to extreme pH *values*

In order to judge correctly the changes in enzymic activity which accompany pH variations, it is necessary to determine whether the enzyme protein has undergone irreversible change of structure. For this purpose the enzyme was kept for 30 min. at extreme pH values; thereafter the pH was readjusted to 8·0 and the recovered activity compared with that of the original preparation. At any pH value between 5·6 and 10·2 the reaction mixture remained perfectly clear and full activity could be restored by readjustment to

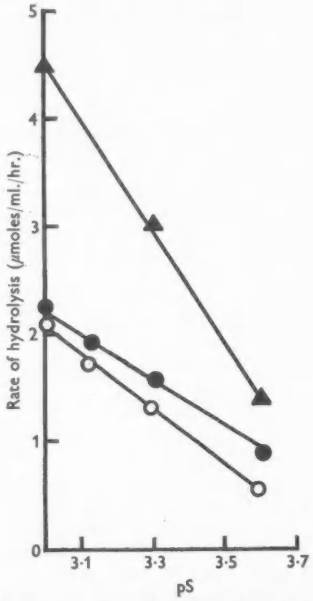

Fig. 1. Hydrolytic rates as function of substrate concentration. Enzyme E-9, after pretreatment with TEPP, was incubated at 0° with 20 μM-CMB for 12 hr., before the addition of the substrate. Final enzyme dilution, 1:100; pH 7·5; temp. 27°. Simultaneously, blanks were run which contained all components except the enzyme, and the enzymic rates were calculated as the difference between total and non-enzymic rates. (The experiment with the *p*-nitro ester was carried out with an enzyme dilution of 1:600, however the rates were recalculated for the purpose of comparison.) All rates represent initial rates, obtained by extrapolation of rate curves to zero time. ●, Phenyl acetate; ○, *p*-methoxyphenyl acetate; ▲, *p*-nitrophenyl acetate.

pH 8·0 (see Table 1). At pH 5·6 the solution becomes just faintly turbid, but clears again when it is brought back to pH 8·0. However, this is not any more the case when the pH is lowered further. Thus the turbidity formed at pH 5·1 does not clear completely when the pH is raised again, and only four-fifths of the original activity can be recovered. In the extreme alkaline range, beyond pH 10·2, no precipitate is formed. Nevertheless, slow irreversible changes take place, and after exposure to pH 10·8 for 15 min. only about 55% of the activity was recoverable.

Enzyme E-9 proved to be more vulnerable (see Table 1). Thus at pH 5·6 one-third of the total activity was lost after exposure for 15 min., and at pH 10·2 almost two-thirds.

pH–*Activity curves of C-esterase*

As spectrophotometric measurements require at least a 1:100 dilution of the present coloured-enzyme preparations, it is often advantageous to increase the activity by the use of a mercurial. Therefore it was necessary to compare the pH dependence of hydrolysis both without and with activator. The curves in Fig. 2 show that for *p*-methoxyphenyl acetate practically identical results are obtained in the presence or absence of sodium *p*-chloromercuribenzoate (CMB). Similar observations were made with phenyl acetate as substrate.

The curves in Fig. 2 represent relative activities as function of pH, the maximum activity of a given preparation serving as 'internal' standard; for example, all rates in the presence of CMB were expressed as percentage of the maximum rate of the enzyme, activated with the same mercurial. If, however, the activation is calculated for each pH as the percentage increase in the non-activated enzyme rate produced by activation, a different picture is obtained (Fig. 3). At low pH values the effect of CMB is practically nil, but it increases sharply when the pH rises to 7·3. Then the slope of the corresponding curve in Fig. 3 diminishes, and the activating effect remains on a nearly constant level up to pH 9·0. Beyond the latter value, the curve rises again.

The points in Fig. 3 represent measurements in which enzyme and activator were kept at pH 7·5 and 4° for 12 hr. Then this mixture was incubated with excess of buffer of the desired pH for 15 min. and finally the substrate added. However, identical results were obtained with the following procedure: enzyme and buffer were incubated at 28° for 15 min. before the mixture of mercurial and substrate was added. This shows that regardless of how the enzyme–activator complex was formed it readjusts itself immediately to a new environment with a different pH.

As shown previously (Bergmann *et al.* 1957), the bond between C-esterase and CMB is very stable, and even after dialysis for 72 hr. only about half of the 'bound' CMB is lost. We have now observed that the same applies to the whole pH range investigated. Accordingly, in short-range experiments dilution of a mixture of enzyme and activator does not influence the degree of activation reached, and it is not necessary (as was done in our early experiments with C-esterase) to add the mercurial to all solutions used subsequently. The changes in activating power as a function of pH, which are demonstrated in Fig. 3, are thus not due

Table 1. *Recovery of enzymic activity after exposure of C-esterase to extreme* pH *values*

The enzyme was kept at the pH indicated. The pH was then readjusted to 8·0 and activity against *p*-nitrophenyl acetate was measured spectrophotometrically. The rates so obtained were expressed as percentage of the rate of hydrolysis by the original enzyme preparation. The temp. was 28°. The same results were obtained, whether the enzyme was activated by CMB or not.

Enzyme	pH	Length of incubation period (min.)	Activity recovered (%)
C-Esterase E-10 (stable to dialysis)	5·1	30	83
	5·6	30	100
	10·2	30	100
	10·8	15	56
C-Esterase E-9 (partly unstable to dialysis)	5·6	15	62, 64
	9·5	15	76
	10·2	15	35, 40

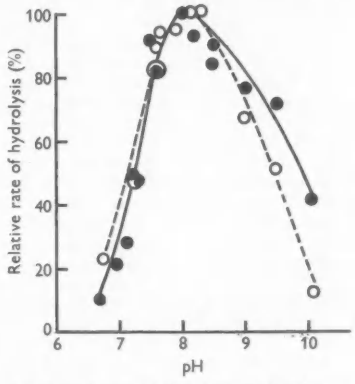

Fig. 2. pH-dependence of the hydrolysis of *p*-methoxyphenyl acetate by C-esterase. ○, Enzyme E-10, 1:50; ●, enzyme E-10, 1:200, after activation with 20 μM-CMB. Substrate was *p*-methoxyphenyl acetate (mM). All rates represent initial rates, calculated as the difference between total and non-enzymic hydrolysis and then expressed as percentage of the maximum rate.

to association or dissociation of enzyme and activator but express structural changes within the stable complex of C-esterase and mercurials.

pH-*dependence of* p-*nitrophenyl acetate*

Unexpected results were obtained with *p*-nitrophenyl acetate. Throughout the whole pH range, the rate increases steadily with increasing pH (Fig. 4), with or without activator. In most experiments it is possible to represent all points by one straight line. It was thought possible that *p*-nitrophenol, one of the hydrolysis products, exerts an activating effect on the enzymic reaction, thus obscuring the expected decline of rates in the alkaline range. This could, however, be excluded by adding free *p*-nitrophenol to the system C-esterase–*p*-methoxyphenyl acetate in the alkaline range, e.g. at pH 9·0. No change of rate was observed. We are thus led to the assumption that OH^- ions themselves enhance the catalytic effect of C-esterase towards the *p*-nitro ester.

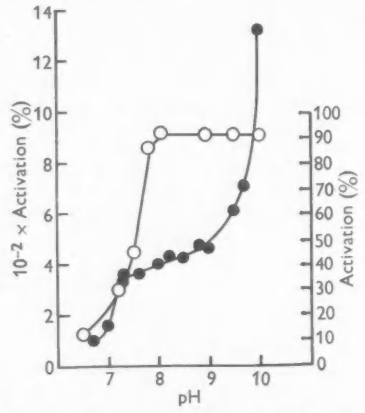

Fig. 3. Influence of pH on the activation of C-esterase by mercurials. Enzyme E-10, pretreated with TEPP, was incubated for 12 hr. at 4° with 20 μM-CMB. The enzyme dilution in this mixture was 1:4. Thereafter 24 vol. of buffer were added and the mixture was left at 28° for 15 min. Finally, the substrate dissolved in an equal volume of water was added (at zero time) and spectrophotometric measurement started at once. Simultaneously, an identical control run was made without activator. Final enzyme dilution for experiments with CMB was 1:200, for control runs, 1:50 (the latter rates were divided by four for comparison). For activation with phenylmercuric acetate, the enzyme, pretreated with TEPP, was dialysed against distilled water for 2 hr. to remove all Cl^- ions, then 0·2 mM-activator was added. Enzyme dilution during incubation at 4° was 1:4. Subsequent procedure was as above, but dilution was effected with 5 vol. of buffer only, so that the final dilution of activated enzyme in this case was identical with that of the control runs (1:50). Final concn. of *p*-methoxyphenyl acetate, mM; temp. 28°. Non-enzymic hydrolysis rate was deducted from the rate measured. Percentage activation was calculated as:

$$100 \times \frac{\text{rate of activated enzyme} - \text{rate of non-activated enzyme}}{\text{rate of non-activated enzyme}},$$

and is plotted as a function of pH. ○, Phenylmercuric acetate (right ordinate); ●, CMB (left ordinate).

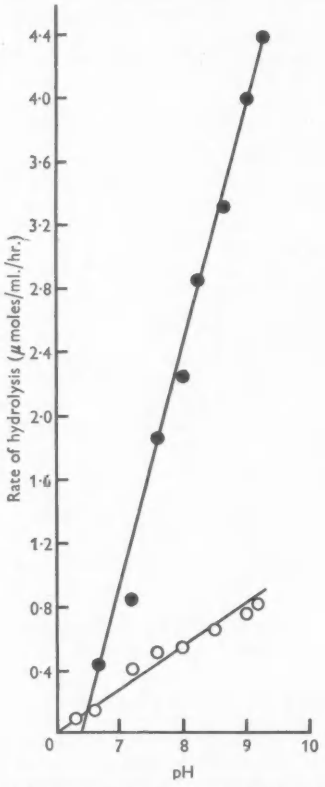

Fig. 4. pH-dependence of the hydrolysis of *p*-nitrophenyl acetate by C-esterase. ○, Enzyme E-10, pretreated with TEPP. Enzyme and buffer were kept at 28° for 15 min. before the substrate was added (at zero time). ●, Enzyme E-10, pretreated with TEPP, was incubated for 12 hr. at 4° with 20 μM-CMB. Thereafter buffer was added and the mixture left for 15 min. at 28°. Finally the substrate was added (at zero time). Final enzyme dilution: without activator, 1:200; with CMB, 1:400. Substrate, mM-*p*-nitrophenyl acetate. All rates represent initial rates and are calculated as total minus non-enzymic hydrolysis.

DISCUSSION

Like other hydrolytic enzymes, C-esterase shows great stability when exposed to acid or alkaline media, which reduce the enzymic rates almost to zero. Within the range pH 5·6–10·2, activity can be recovered quantitatively by readjustment of the pH to its optimum value.

All known esterases possess a more or less well-defined pH optimum of activity, which serves as expression of the dual mechanism operative in enzymic hydrolysis (Bergmann *et al.* 1958), i.e. simultaneous catalysis by a nucleophilic and an electrophilic group, the latter acting probably through hydrogen bonding. Accordingly, the pH–activity curves in Fig. 2 may be interpreted as indicating the possible presence of an imidazol group, which may be responsible for the increase in rates when the pH rises from 5·5 to 8·0. Likewise, an electrophilic component of the esteratic site must be responsible for the decay of activity when the pH rises from 8 to 10. The active centre of C-esterase is thus similar, in principle, to that of A- and B-esterases. However, the curves in Fig. 2 differ in shape from the curves reported for eel esterase with the same substrates. With the latter enzyme, a plateau was found between pH 7·0 and 9·5. Only above pH 9·5 did the decay of enzymic activity become measurable (Bergmann *et al.* 1958). With C-esterase, on the other hand, a sharp optimum is apparent at pH 8·0 and above this value the rates decrease rapidly. The electrophilic group in the esteratic site is thus presumably different in the two enzymes.

C-Esterase differs markedly from other esterases in its susceptibility to activation by organic mercurials. For example, cholinesterases are very little influenced by CMB (Mounter & Whittaker, 1953), and DFPase is strongly inhibited (Mounter, Floyd & Chanutin, 1953). The variation of C-esterase activation with pH changes sheds light on the character of the enzyme–activator complex. This complex is rather stable throughout the whole pH range of the present studies, as evidenced by the rapid return of the activating effect when the pH is changed and then restored, and also by dialysis experiments. Apparently the mercurials are firmly bound to a group which does not change its binding power markedly with pH. In addition, they must combine with a group in the active centre, which becomes 'inactivated' with decreasing pH. A reasonable assumption would be that an imidazol group is present, which in its basic form complexes the organic mercuric ion, but is unable to do so when converted into the imidazolinium cation. The left-hand portion of both curves in Fig. 3 indicates indeed a group with pK 7·0–7·5. The carboxyl group in CMB enhances the activating effect above pH 8·0, as becomes evident by comparison of the curves in Fig. 3. Since in this pH range the carboxyl is completely ionized, the difference between CMB and phenylmercuric ion is probably to be ascribed to a change in the active centre itself.

The bond between C-esterase and the postulated dialysable cofactor (Bergmann *et al.* 1957) would be of a different character. The cofactor appears to be easily lost by pH changes in either direction. In addition, CMB and phenylmercuric ion activate also C-esterase with cofactor, so that the native activator would have to be bound to a group different from that to which the mercurials become attached.

An interesting problem is posed by the curious pH–activity curve found for the system C-esterase–*p*-nitrophenyl acetate (Fig. 4). The following observations should be borne in mind. Eel esterase splits this substrate at a constant rate throughout the range pH 7·0–9·5 (Bergmann *et al.* 1958). On the other hand, the rate of chemical hydrolysis of the *p*-nitro ester by OH^- ions increases with pH. The increase is much greater than the corresponding rise in hydrolytic rate observed in the alkaline pH range for the system C-esterase–*p*-nitrophenyl acetate. This relationship is clearly demonstrated in Fig. 5, in which the two last-mentioned reaction rates are plotted as functions of OH^- ion concentration. It is suggested therefore that, in addition to the catalytic effect of OH^- ions, another factor comes into play which changes the reaction order with regard to OH^- ion and thus diminishes the increase in rate with increasing pH. We may, for example, assume that the change of rate of hydrolysis of the *p*-nitro ester with increasing pH is the resultant of two effects: (*a*) a decreasing catalytic influence of the enzyme analogous to the decline of activity in the alkaline range shown in Fig. 2; (*b*) an increasing catalytic effect of OH^- ion.

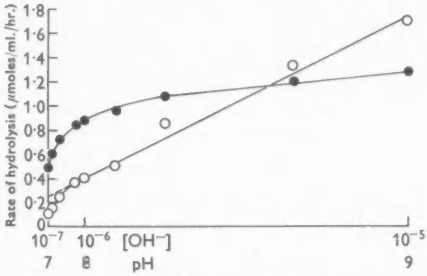

Fig. 5. Rate of hydrolysis of *p*-nitrophenyl acetate as a function of hydroxyl ion concentration. ○, Non-enzymic hydrolysis; ●, hydrolysis by enzyme E-9, 1:100 (total minus non-enzymic rate). Ester concn. mM; temp. 28°; all rates plotted represent initial rates.

SUMMARY

1. Purified C-esterase (from hog's kidney) is stable within the range pH 5·6–10·2. After exposure of the enzyme to these extremes of pH, its activity can be fully recovered by readjustment of the pH to 8·0.

2. The activating effect of organic mercurials is pH-dependent. The changes are immediate and completely reversible.

3. Some of the preparations of C-esterase appear to contain a dialysable cofactor, which is lost rapidly by exposure of the preparations to pH 5·6 or to pH 10·2.

4. The pH–activity curves for phenyl acetate and its *p*-methoxy derivative exhibit an optimum around pH 8. In this respect, C-esterase behaves similarly to esterases of the A- and B-type.

5. In contrast, the rate of the enzymic hydrolysis of *p*-nitrophenyl acetate increases steadily up to the experimental limit of pH 9·5.

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Condensed Tannins

2. BIOGENESIS OF CONDENSED TANNINS BASED ON LEUCO-ANTHOCYANINS*

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The previous work by Roux (1957*a*, *b*, 1958*a*) and by Roux & Evelyn (1958) showed the association of monomeric and complex leuco-anthocyanins of similar structural pattern in those typical 'condensed tannins' present in extractives of black-wattle bark and quebracho heartwood. Chromatographic evidence illustrated the apparent conversion of monomeric into the complex polymeric leuco-anthocyanins. The present study provides details of the chromatographic identification, isolation and characterization of some of these new monomeric leuco-anthocyanins. By means of a study of molecular weight evidence is also provided to support the concept of their conversion into complex tannins during radial translocation within the plant.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Analyses of C, H, methoxyl and acetyl are by Weiler and Strauss, Oxford. Tannin analyses by the hide-powder (shake) method are by Miss S. Whitelaw of these Laboratories.

* Part 1. Roux & Evelyn (1958).

Variations in quebracho woods. The wood of *Schinopsis balansae* from Vera, Santa Fe, and also from unknown areas in the Argentine, appeared uniformly dark-reddish brown in cross-section, without differentiation into heartwood and sapwood areas. A single specimen obtained recently from the same source was clearly divided into a white sapwood and a light-amber heartwood.

A cross-section of *Schinopsis quebracho-colorado* [syn. *S. lorentzii*; see Barkley & Meyer (1950) for discussion on nomenclature] collected from Atamisqui, Santiago del Estere, (specimen A) showed sharp subdivision into a yellow sapwood and a deep-amber, almost black, central-heartwood region (Roux, 1958*a*). Other samples obtained from the same source but from unknown regions of the Argentine, and also from the Union Department of Forestry plantations at Hanglip, Northern Transvaal (specimens B), differed in that a white sapwood 2 in. wide surrounded the light amber–brown central heartwood. These are almost identical in appearance with the divergent sample of *S. balansae*.

Examination of extractives of Schinopsis *spp.*

Isolation of a new leuco-fisetinidin. Dry wood (100 g.) of *S. quebracho-colorado* (specimen A, a sapling of 7–8 cm. diam.) was cut into chips and ground into a fine powder with a Wiley mill. The powder was extracted first with

1·05 l. of methanol (three extractions with 350 ml. each for 24 hr.), and finally exhaustively with 1·75 l. of acetone–water (1:1) (five extractions for 48 hr. each). The extracts were streaked on to paper-chromatographic sheets (Whatman no. 3) and developed with 2% acetic acid. The monomeric leuco-anthocyanin migrated in advance of the polymeric units (Roux & Evelyn, 1958), and could be located by spraying a strip cut from the edge of the sheet with the toluene-*p*-sulphonic acid reagent. Subsequently it was located merely by reference to certain fluorescent bands on paper sheets. The bands were cut out and eluted with 70% ethanol. From a concentrate of the eluents the leuco-anthocyanin crystallized easily in long thin needles (2 g.). Simultaneous elution of the tannins with lower R_F values showed that the leuco-anthocyanin formed 10% of the tannins present, and the yield was also 2% on wood weight.

The compound, which is sparingly soluble in cold water, was recrystallized from water as a white dihydrate (Found: C, 55·8; H, 5·6; loss at 110°, 11·0. Calc. for $C_{15}H_{14}O_6,2H_2O$: C, 55·2; H, 5·6; H_2O, 10·6%), m.p. 130° with reddening after softening at 110°. A green colour with $FeCl_3$ indicated the presence of a catechol nucleus. On paper chromatograms its R_F in 2% acetic acid (0·50) and scarlet colour developed with toluene-*p*-sulphonic acid reagent suggested the presence of a flavan-3:4-diol structure (Roux, 1958*b*; Roux & Evelyn, 1958). In a partitioning mixture, e.g. butan-1-ol–acetic acid–water (4:1:5), it migrated at the same rate (R_F 0·76) as (+)-catechin, indicating the probable presence of the same number of hydroxyl groups on the C_{15} skeleton.

The compound was easily converted into the anthocyanidin, fisetinidin, in the presence of 3 N-HCl in propan-2-ol solution under pressure at 100°. The anthocyanidin was identified by the methods previously outlined (Roux, 1957*a*, *b*; Roux & Evelyn, 1958). The comparative yields of fisetinidin formed from similar leuco-anthocyanins are represented in Table 1. Fusion with KOH under controlled conditions (Roux, 1952) resulted in degradation into β-resorcylic and protocatechuic acids.

The leuco-fisetinidin from quebracho wood shows the same absorption maximum (282 mμ) as the 7:3′:4′-trihydroxyflavan-3:4-diol obtained by the catalytic (platinum oxide) hydrogenation of fustin (Freudenberg & Roux, 1954; Roux & Freudenberg, 1958), and also a similar $\epsilon_{max.}$ (6521) to this synthetic flavan-3:4-diol (6414). Its specific rotation in methanol, $[\alpha]_D^{25} -11·1 \pm 1·1°$ (*c*, 1·08) distinguished it from (+)-mollisacacidin (Keppler, 1957) and also from the hydrogenation product of fustin $[\alpha]_D^{23} -2·4°$ (Roux & Freudenberg, 1958). Anhydrous (−)-leuco-fisetinidin readily reabsorbs moisture from the atmosphere, and drastic drying under vacuum at 110° over P_2O_5 is required for the removal of water of crystallization. The compound assumes a pink colour under these conditions (Found: C, 61·6; H, 5·0. Calc. for $C_{15}O_{14}O_6$: C, 62·1; H, 4·9%).

7:3′:4′-Trimethoxyflavan-3:4-diol. (−)-Leuco-fisetinidin (500 mg.) in 50 ml. of methanol at −5° was methylated with 45 ml. of ethereal diazomethane generated from 10 g. of nitrosomethylurea. The mixture was kept at −5° overnight, and reduced to small volumes by evaporation and poured into water. The white product (0·4 g.) failed to crystallize from conventional media such as absolute ethanol or methanol, and settled out as a sludge on cooling. The compound crystallized as a hydrate in fine needles (m.p. 81–86°) from 50% aq. ethanol. After drying overnight over silica gel the hydrate contained 1·5 mol. prop. of water (Found: C, 60·0; H, 6·6; OMe, 25·9. Calc. for $C_{18}H_{20}O_6,1·5H_2O$: C, 60·2; H, 6·3: OMe, 25·9%). One mol. prop. of water was easily lost by drying for 2 hr. at 60° (Found: C, 63·2; H, 6·2. Calc. for $C_{18}H_{20}O_6,0·5H_2O$: C, 63·3; H, 6·2%). The remaining water was strongly held, and drying for 6 hr. at 70° over P_2O_5 was required to give the anhydrous form. (Found: C, 65·1; H, 6·2; OMe, 28·0. Calc. for $C_{18}H_{20}O_6$: C, 65·1; H, 6·1; OMe, 28·0%). The anhydrous trimethylether settles slightly at 81–84°, sharply at 129° and the melt liquefies completely at 134°. Under the conditions of Pigman, Anderson, Fischer, Buchanan & Browning (1953), and in the presence of HCl, the trimethoxyflavan-3:4-diol is converted into a scarlet pigment, presumably 3-hydroxy-7:3′:4′-trimethoxypyrilium chloride, and the corresponding flavonol (paper chromatogram).

3:4-Diacetoxy-7:3′:4′-trimethoxyflavan. The trimethylether (500 mg.) was dissolved with warming in 5 ml. of acetic anhydride, and 1 g. of freshly dried sodium acetate added. The mixture was slowly brought to the boil, and kept near the boil for 5–10 min. It was then cooled, and poured into 50 ml. of iced water and left overnight. The dry solid (0·45 g.) was repeatedly recrystallized from ethanol. The product had m.p. 102–104° (Found: OMe, 22·8; $CO.CH_3$, 20·9. Calc. for $C_{22}H_{24}O_8$: OMe, 22·4; $CO.CH_3$, 20·7%).

3:4:7:3′:4′-Penta-acetylflavan. (−)-Leuco-fisetinidin dissolved in 5 ml. of pyridine was treated with 5 ml. of acetic anhydride. Reaction occurred spontaneously at room temperature, and after 2–3 hr. the mixture was poured into iced water. A pure white granular product (0·53 g.) separated, which failed to crystallize from ethanol, methanol or other solvents (Found: C, 59·7; H, 5·1; $CO.CH_3$, 41·9. Calc. for $C_{25}H_{24}O_{11}$: C, 60·0; H, 4·8; $CO.CH_3$, 43·0%).

Table 1. *Yields of fisetinidin from monomeric leuco-fisetinidins*

Source	Weight of leuco-anthocyanin (mg.)	Yield of fisetinidin chloride (%)
Schinopsis quebracho-colorado	3·5	15·7
	2·4	17·3
Acacia mollissima	3·2	20·0
	4·7	13·2
Reduction product of fustin	6·9	14·5
	4·8	13·6

The extractives of woods of *Schinopsis* spp. were initially examined for leuco-anthocyanins by two-way paper chromatography and the toluene-*p*-sulphonic acid-spraying reagent (Roux, 1957*b*). Chromatograms of *S. quebracho-colorado* showed the presence of a monomeric leuco-fisetinidin in high concentration in the sapwood of specimen A, and in the light-amber heartwoods of specimens B. Complex leuco-fisetinidins were present in all specimens. The wood of the uniformly dark-coloured samples of *S. balansae* contained complex leuco-fisetinidins of similar distribution to those

present in *S. quebracho-colorado*. The divergent sample of *S. balansae* also contained monomeric leuco-fisetinidin at the sapwood–heartwood junction, as also recorded by King & White (1957*a*, *b*) for both species of *Schinopsis*.

In the adult trees, of about 35 cm. diameter, of *S. quebracho-colorado* and *S. balansae* which contain outer sapwood 4–5 cm. wide, the leuco-fisetinidin is present in the sapwood only in low concentration, and in the outer margin of the heartwood, 1 cm. wide, in high concentration. The central heartwood of about 22 cm. diameter contains only polymeric condensed tannins, amongst which complex leuco-fisetinidins are present, and overall the percentage of monomeric leuco-fisetinidins is therefore much lower than in the sapling examined.

The outer sapwood (Mezey, 1947; Howes, 1953) and also the heartwood edge (in a typical log received by us from the Argentine) is removed from the heartwood core before commercial extraction. This is probably one explanation of the complete absence of monomeric leuco-fisetinidin in the commercial extract (see Freudenberg & Maitland, 1934; Roux, 1958*b*). The uniformly dark-coloured specimens of *S. balansae*, showing no sapwood, contained typical polymeric but no monomeric forms (Roux, 1958*a*) and may represent varietal forms of the same species.

Radial sampling of S. quebracho-colorado *heartwood*. The heartwood (specimens B) was sampled by drilling (Fig. 1), the concentration of polyphenolics being very low in the sapwood. The wood of these specimens also showed the radial increase in complexity previously found in specimen A (Roux, 1958*a*), although emphasis on the polyphenolic tannins of low R_F (zero R_F in 2 % acetic acid) in the central heartwood was not quite as pronounced as in specimen A. The percentages of extractives and 'non-tannins' of the radial samples, the yield of fisetinidin from each and the average molecular weight of the tannins (Evelyn, 1954*a*, *b*; Roux & Evelyn, 1958) were estimated (Table 2).

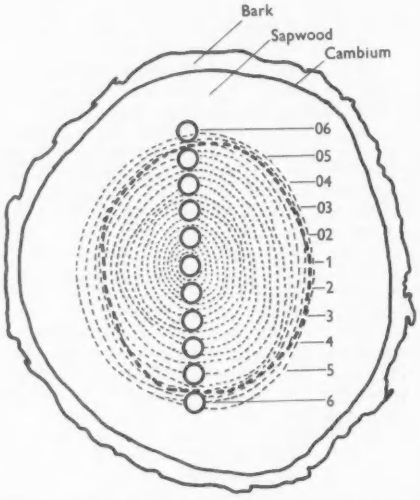

Fig. 1. Sampling positions on the cross-section of the wood of *Schinopsis quebracho-colorado*. Samples were obtained by drilling. Drawn to scale, to illustrate the annual rings (light circular broken lines) within the 'heartwood' area (heavy circular broken line).

Table 2. *Non-tannin content, yields of anthocyanidins and average molecular weights of tannins present in radial samples from the wood of* Schinopsis quebracho-colorado

See Fig. 1 for sampling position. Percentage of extractives is based on dry wt. of wood samples. Wood extracts 06 and 6 contain mainly sugars and simple phenolic constituents. The percentage of 'tannins' in the extractives may be obtained by subtraction of the value for 'non-tannins' from 100 %. Variable results for conversion into anthocyanidins are due to a variety of known and unknown factors influencing the generation reaction (Pigman *et al.* 1953). For central-heartwood samples 04–4, where monomeric leuco-anthocyanins are largely absent, the conversion based on 'tannin' content varies within limits 4·2–8·2 %. Calculations are based on yield of fisetinidin from anhydrous extractives (see Roux & Evelyn, 1958).

Sampling position	Extractives (%)	Non-tannins (% of extractives)	Conversion into anthocyanidins (%)	Average mol.wt. of tannins
06	3·6	—	0·5	—
05	21·3	25·0	4·2	655
04	22·3	17·2	5·5	726
03	24·4	15·1	4·4	801
02	25·3	10·6	3·8	953
1	20·0	9·9	4·5	1039
2	22·1	11·8	6·3	919
3	22·0	15·1	5·5	846
4	20·4	20·6	6·5	751
5	9·8	39·0	4·6	659
6	3·2	—	1·4	—

Leuco-anthocyanins of the bark of Schinopsis quebracho-colorado. The methanol extractives of the dried bark gave an intense scarlet coloration with HCl, under the conditions of Pigman *et al.* (1953), which was due to delphinidin, and from two-way paper chromatograms of the extracts complex leuco-delphinidins of low R_F appeared to be present in addition to mobile non-leuco-anthocyanin constituents of higher R_F.

Examination of extractives of Acacia *spp.*

Monomeric leuco-anthocyanins of black-wattle-bark extractives. Freshly stripped black-wattle (*Acacia mollissima*) bark was sliced into thin slivers with a stainless-steel knife, and extracted at 0–5° with methanol. The methanolic extract was streaked on to sheets of Whatman no. 3 paper, and the chromatogram was developed with 2% acetic acid. Strips cut from each sheet, corresponding to R_F 0·50–0·60, were eluted with 70% ethanol. The concentrated eluent was examined by two-dimensional chromatography with butan-2-ol saturated with water and then with 2% acetic acid. Of the reducing areas present in this fraction only two, LF (R_F 0·68 and 0·50 in the solvents respectively) and LR (R_F 0·54 in each solvent) gave the scarlet leuco-anthocyanin reaction with the toluene-*p*-sulphonic acid reagent (Roux, 1957*b*). The $FeCl_3$ spray gave a green coloration with LF (catechol unit), whereas LR afforded a blue coloration (pyrogallol unit). The position of LF corresponded exactly with leuco-fisetinidin or 7:3′:4′-trihydroxyflavan-3:4-diol, and LR with leuco-robinetinidin or 7:3′:4′:5′-tetrahydroxyflavan 3:4-diol on paper chromatograms, when artificial mixtures of these reference compounds (Freudenberg & Roux, 1954; Roux & Freudenberg, 1958) were made with this wattle fraction.

The wattle fraction obtained above (R_F 0·5–0·6 in 2% acetic acid) was streaked on Whatman no. 3 paper, and the chromatogram was developed with butan-1-ol–acetic acid–water (12:3:5, by vol.). After development, fractions corresponding with LF and LR were cut. From the LF fraction fisetinidin and from the LR fraction robinetinidin were generated by treatment with HCl (Pigman *et al.* 1953) and identified as before. The monomeric leuco-fisetinidin and leuco-robinetinidin constitute some of the minor constituents of wattle extract, and their concentration is considered too low to permit isolation in sufficient quantity for detailed study.

Leuco-anthocyanins of the bark of Acacia melanoxylon. The methanolic extractives of the fresh bark gave the same results as those given by the dried bark of *S. quebracho-colorado* (see above).

DISCUSSION

The (−)-7:3′:4′-trihydroxyflavan-3:4-diol from the sap- and heart-woods of *Schinopsis quebracho-colorado* differs in its melting-point and the melting-points of its derivatives from the (+)-leuco-fisetinidin (mollisacacidin) obtained by Keppler (1957) from the heartwood of *Acacia mollissima*, and also from the hydrogenation product of dihydrofisetin (Roux & Freudenberg, 1958). These leuco-fisetinidins, on the other hand, show similar behaviour in the way in which they redden at and above 110°, and in the non-crystalline nature of their penta-acetyl derivatives. The (−)-leuco-fisetinidin from quebracho wood was also characterized by Freudenberg & Weinges (personal communication from Professor K. Freudenberg), and was noticed by King & White (1957*a*, *b*), although their tentative identification of this constituent as an amorphous monoglucoside is not exact. The trimethyl derivative, which requires the presence of moisture in order to promote crystallization, differs markedly in this respect from the similar derivatives of other leuco-fisetinidins.

The rapid genesis of the monomeric leuco-fisetinidin (also recorded by King & White, 1957*a*, *b*) in the sapwood edge (specimen A), or in the sapwood and sapwood–heartwood junction (specimens B) of *S. quebracho-colorado* and in the sapwood–heartwood area of one specimen of *S. balansae*, and its rapid and progressive transformation into condensed tannins of the leuco-fisetinidin type in the central-heartwood area, provides presumptive evidence of the biogenesis of complex tannins from monomeric leuco-anthocyanins (Roux, 1957*a*, *b*; 1958*a*), and strongly suggests that the long-sought precursor of the quebracho tannins is, in fact, leuco-fisetinidin (Roux, 1958*b*). A study of the radial samples of *S. quebracho-colorado* by molecular-weight methods (Table 2) shows that the transition of monomeric leuco-fisetinidin into progressively more complex leuco-fisetinidins from the outer to the central heartwood is accompanied (*a*) by a gradational increase in molecular weight from 650, for the outer heartwood, to 1050, for the central heartwood, and (*b*) by a progressive reduction in the non-tannins present, or by an increase in the tannin:non-tannin ratio, but with no definite trend in the total percentage of extractives present in each sample. The percentage of anthocyanidins generated from quebracho tannins (Table 2) compared with the yield from monomeric leuco-fisetinidins (Table 1) indicates a total leuco-anthocyanin content of 20–35% in *S. quebracho-colorado*. The conversion of monomeric into polymeric leuco-anthocyanins is therefore one of the important transformations which occurs in what is obviously a complex mixture of tannin constituents

(see King & White, 1957*a*, *b*). The high percentage (10%) of monomeric leuco-fisetinidin present in the outer heartwood- or sapwood-tannin mixture, and the relative completeness of its transformation into complex leuco-anthocyanin tannins in the central heartwood (Roux, 1958*a*), also supports this view. Carbohydrate non-tannins and also polyphenolic half-tannins (see Roux, 1957*c*) are apparently converted into tannins during this process (Table 2).

In most barks, e.g. black-wattle bark (*A. mollissima*), a greater degree of differentiation and complexity in cell structure exists, and attempted layerwise radial sampling from the cambial to cortical layers did not show the transformations evident in some heartwoods. It is, nevertheless, interesting to record the association of monomeric and complex leuco-anthocyanins of the robinetinidin and fisetinidin types in the whole fresh-bark extract of *A. mollissima*.

Although heart-wood formation is a complex phenomenon, and heartwoods are somewhat different anatomically in the angio- and gymnosperms, Erdtman (1952, 1955, 1956) has found that amongst the conifers, heartwood formation is controlled from the cambium. Heartwood formation ceases, for example, on injury of the cambium and is re-established once the cambial layer has healed. Furthermore, heartwood and bark constituents appear to originate in the cambium and are distributed both centripetally (via medullary rays) and centrifugally in many instances. Hillis (1956*a*, *b*) described identical concepts for various *Eucalyptus* species, where the leuco-anthocyanins of the xylem are considered to originate in the leaves, and to represent intermediate precursors of heartwood and bark extractives. King & White (1957*a*, *b*) concluded that the 'hydrolysable' tannins of the leaves of *Schinopsis* spp. constitute the phenolic raw material, which is ultimately converted into typical 'condensed' tannins of the heartwood. Catechin and leuco-anthocyanins participate in this conversion. They, however, overlooked (White, 1956, 1957; King & White, 1956) the leuco-anthocyanidin nature of some of these condensed tannins (see Roux, 1957*a*, *b*; 1958*a*).

Considering only differences in the leuco-anthocyanins present in the barks and heartwoods of *Schinopsis* spp. (leuco-delphinidins and mainly leuco-fisetinidins, Roux, 1957*a*), of *Acacia melanoxylon* (leuco-delphinidins and melacacidins, King & Bottomley, 1954) and of *A. mollissima* and closely related species (leuco-robinetinidins + leuco-fisetinidins and mollisacacidin, Keppler, 1957), it appears that precursor material originating from the cambium is subjected to the action of two different and highly specific enzymic systems in the heartwood and in the bark, after centripetal and centrifugal translocation. These enzymes vary in activity, e.g. monomeric melacacidins and mollisacacidins persist into the central heartwoods of *A. melanoxylon* and *A. mollissima* respectively, whereas in heartwoods of *Schinopsis* rapid conversion of the monomeric leuco-fisetinidin occurs, and also the polymeric forms appear to undergo conversion (Table 2 and Roux, 1958*a*). This rapid enzymic conversion persists throughout the heartwood of *S. quebracho-colorado*, showing that in this instance even the central-heartwood cells still contain active-enzyme systems. Differences between the high average molecular weights (1460: ranging from 700 to 2300, Roux & Evelyn, 1958) of commercial quebracho extract from adult (100–175 years) trees, and the much lower average (650–1050) from the young tree (42 years) examined, suggests that progressive molecular-weight increases in the central heartwood occurs even during very advanced stages of growth in *Schinopsis* spp. The shape of the heartwood in *S. quebracho-colorado* usually follows the shape of the cambium rather than that of the annual rings. The concept of the cambium as common origin of heartwood and bark tannins finds support in the observations that whereas the cambium of *A. mollissima* contains no tannins (Sidey, 1953), both the oldest and youngest strippable barks of *A. mollissima* contain complex tannins of almost equally high average molecular weights (Evelyn, 1956).

The above instances of the association of monomeric ('non- or half-tannins') and complex leuco-anthocyanins ('condensed tannins') in commercially important vegetable extracts used for tannage, must be linked directly with the high correlation between the occurrence of leuco-anthocyanins and 'botanical' tannins in nature, and with their exceptionally wide distribution (Bate-Smith & Lerner, 1954; Bate-Smith, 1957).

SUMMARY

1. Details are given of the isolation and characterization of a monomeric (−)-leuco-fisetinidin from the wood extractives of *Schinopsis quebracho-colorado*, and of the identification by chromatography of a monomeric leuco-robinetinidin and a leuco-fisetinidin in the fresh-bark extract of *Acacia mollissima* ('wattle' extract).

2. Differences in bark and heartwood leuco-anthocyanins in these and other species suggest that the cambium furnishes the common precursors of both bark and heartwood tannins, and that different and very specific enzymic systems effect conversion into condensed tannins in each portion of the plant.

3. Evidence for the biosynthesis of condensed tannins from monomeric leuco-anthocyanins is presented.

4. Conversions persist into the central heartwood, suggesting that these cells still contain active-enzyme systems at an advanced age, even after heavy deposition of tannin.

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Studies in the Bile Acids

3. THE CONJUGATED BILE SALTS OF CERTAIN PRIMATES*

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In a previous paper, the non-ketonic acids of human bile were described (Wootton & Wiggins, 1953). The major constituent was shown to be chenodeoxycholic acid (3α:7α-dihydroxycholanic acid) and it was suggested that the presence of a high proportion of this acid might be characteristic of the human species. To test this suggestion, a series of primate biles have now been examined.

The human-bile analyses reported in 1953 were done by hydrolysing the conjugated bile salts and submitting the bile acids produced (after methylation) to chromatographic separation and identification by infrared spectroscopy. No distinction was therefore made between glycine-conjugated and taurine-conjugated acids. However, Ahrens & Craig (1952) have developed a countercurrent method capable of separating these two classes of conjugates and this procedure has been applied as the initial stage of the primate-bile analyses. The separated fractions of the bile have then been carried separately through the remainder of the analysis. This complete method has been applied to samples of gall-bladder bile derived from the human (three specimens), the chimpanzee (*Pan satyrus*), the white-nosed monkey (two specimens of *Cercopithecus nictitans mortinii*), the mona monkey (*Cercopithecus mona*) and the tantalus guernon (*Cercopithecus aethiops tantalus*). The specimens of monkey bile were kindly provided by Professor G. A. D. Haslewood.

* Part 2: Wootton & Wiggins (1953).

EXPERIMENTAL

Material. Bile from all species was obtained at autopsy within a few hours of death. The contents of the gall bladder were mixed with 2 vol. of ethanol, allowed to stand for 24 hr., filtered and evaporated to dryness.

Countercurrent separation. The apparatus used was an all-glass system with a maximum capacity of thirty tubes. Each tube required 40 ml. of upper phase and 40 ml. of lower phase. When a transfer was done, the upper phases moved while the lower phases remained in the same tubes. The separation was done in two stages. The first stage removed the cholesterol and lipid material, leaving purified bile salts. The solvent system consisted of 97·5% (v/v) acetic acid–heptane (Ahrens & Craig, 1952), shaken together and allowed to separate. Six tubes were filled with lower phase, and the ethanol-soluble bile fraction (about 1 g.) was dissolved in the first tube. Fifteen upper phases were then passed through the apparatus. The contents of the first four tubes were combined and evaporated to dryness, yielding purified bile salts.

The second stage separated the glycine conjugates from the taurine conjugates. The solvent system was 10% (v/v) acetic acid (lower phase) equilibrated with an equal volume of heptane–butanol (70:30, v/v) (upper phase). Nine tubes were filled with lower phase and the purified bile salts were dissolved in the first tube. Twenty-five upper phases were then passed through and transfers continued until all the upper phases had left the tubes. The first 22 upper phases, containing the glycine-conjugated fraction, were combined and evaporated to dryness. The contents of the first six tubes were combined and dried to give the taurine-conjugated fraction.

Test of countercurrent separation. It was considered advisable to test both stages of the separation. This was done for the initial purification by submitting a bile specimen to an extended experiment with a larger number of tubes. A quantity (0·9 g.) of the ethanol-soluble material from bile sample 2 (Table 1) was distributed between 30 tubes by using the acetic acid–heptane system. The upper and lower phases of each tube were separately evaporated to dryness and tested for the presence of cholesterol by the Liebermann–Burchard reaction (King, 1951) and cholic acid (3α:7α:12α-trihydroxycholanic acid) by a modified Pettenkofer reaction (see below). Positive results are shown in Table 1.

The second stage of the separation was tested similarly by distributing 1·07 g. of purified bile salts from sample 1 between 30 tubes by using the acetic acid–heptane–butanol system. The bile salts were dissolved in the lower phase of the first tube and 33 upper phases were passed successively into the system. The three upper phases which passed through the last tube were collected together. The contents of the various tubes were separately evaporated to dryness and a portion of the material in each tube was tested for cholic acid by the modified Pettenkofer reaction. Another measured portion (1 mg.) was dissolved in 5 ml. of 6N-hydrochloric acid, heated at 100° for 24 hr. and evaporated to dryness in a current of air. Water (2 ml.) was then added and evaporated; this procedure was repeated twice more to remove traces of hydrochloric acid. The hydrolysed residue was dissolved in a few drops of water and examined for amino acids by ascending paper chromatography, with a collidine–lutidine–water solvent and ninhydrin spray. Markers of glycine and taurine were run with each chromatogram.

In preliminary experiments, as many as six ninhydrin-reacting spots were seen. To remove free amino acids present in the purified bile salts, the hydrochloric acid used in the hydrolyses was therefore shaken with solid silver nitrite. Results obtained after this procedure are shown in Table 2. There is a marked discontinuity in the distribution of Pettenkofer-reacting material, and the taurine conjugates are confined to the first three tubes. However, glycine, which must have been present as conjugates in the purified bile salts, appears in all tubes. This is not surprising since many substances are conjugated with glycine.

Table 1. *Test of first stage of countercurrent separation*

Bile sample 2; 0·9 g. of ethanol-soluble material. Solvent system: 97·5% (v/v) acetic acid–heptane. The figures in the fourth column are the approximate weights derived from the individual fractions before combination and final drying.

Lower phase no.	Liebermann–Burchard reaction	Pettenkofer reaction	Wt. (mg.)
1	Negative	Positive	800
2	Negative	Positive	
3	Negative	Faintly positive	
4–8 (combined)	Negative	Negative	7
Upper phase no.			
15–12 (combined)	Negative	Negative	27
11–9 (combined)	Faintly positive	Negative	15
8–1 (combined)	Positive	Negative	245

Table 2. *Test of second stage of countercurrent separation*

Bile sample 1; 1·07 g. of purified bile salts. Solvent system 10% (v/v) acetic acid/heptane–butanol (70:30, v/v).

Tube no.	Pettenkofer reaction	Amino acids detected	Wt. (mg.)
1	Positive	Glycine + taurine	531
2	Positive	Glycine + taurine	76
3	Faintly positive	Glycine + taurine	30
4	Negative	Glycine	20
5–13 (combined)	Negative	Glycine	54
14	Faintly positive	Glycine	2
15–30 (combined)	Positive	Glycine	139
Upper phases 1–3	Positive	Glycine	167

From the results of these tests, the abbreviated procedure described above was devised. Final tests with mixtures of pure synthetic glyco- and tauro-cholic acid showed that separation was complete and recoveries of over 90% were obtained.

Further stages in analysis. The glycine- and taurine-conjugated fractions were separately hydrolysed, methylated, chromatographed on silica-gel columns and examined in the infrared spectrophotometer as described by Wootton & Wiggins (1953).

Modified Pettenkofer reaction. The reagent was prepared by cooling an aqueous solution of fructose (1 mg./ml.) in ice–water and adding dropwise an equal volume of concentrated sulphuric acid with constant mixing and cooling. The reagent remained colourless for several hours at 0°. A solution of the material to be tested (1 mg.) in 2N-NaOH (2 ml.) was mixed with 2 ml. of reagent, heated for 2 min. in a boiling-water bath and cooled immediately. A simultaneous reagent blank was carried through and another similar portion of the material was treated with aq. 50% (v/v) H_2SO_4 in place of the reagent.

The reagent blank was clear-yellow in colour, while the sulphuric acid tube usually showed slight charring. If the material contained cholic acid or its conjugates, the test solution had a cerise-red colour. When conducted in this way, the test was negative with lithocholic acid (3α-hydroxycholanic acid), deoxycholic acid (3α:12α-dihydroxycholanic acid) and chenodeoxycholic acid.

RESULTS

The complete analyses of the eight bile samples are presented in Table 3. The results are expressed as percentages calculated from the isolated methyl esters. In all species, the major part of the bile acids was found to be a mixture of cholic acid, chenodeoxycholic acid and deoxycholic acid, but minor components were also occasionally detected. Lithocholic acid was present in the bile of *Cercopithecus mona* and a keto acid, 3α:12α-dihydroxy-7-oxocholanic acid, was found in *C. aethiops tantalus* and *C. nictitans mortinii*.

DISCUSSION

When the glycine- and taurine-conjugated fractions of a single bile sample are compared, the proportions of the major constituent acids are the same within experimental error. An exception is specimen 7, which was available only in very small amount; the results are therefore subject to a large experimental error. The proportion of taurine conjugates varies from 10 to 43% of the whole. Rosenthal & Falkenhausen (1923), as a result of the study of normal and pathological biles, postulated that the proportion of each conjugate present depends on the availability of taurine, which is used preferentially. The present results do not conflict with this view.

The position is probably the same with regard to the minor constituents, lithocholic acid and 3α:12α-dihydroxy-7-oxocholanic acid. These acids were detected only in the glycine fractions; if they were present in the same proportion in the taurine fractions, the absolute amount would have been below the lower limit of detection of the method. The quantity of material required to produce a spectrum which can be identified depends somewhat on the purity of the sample and in particular on the completeness with which it has been separated from other bile acids. In favourable circumstances rather less than 1 mg. is needed.

Table 3. *Complete analysis of bile salts of eight primate biles*

Sample	Species	Wt. of purified salts (g.)	Conjugate fraction (% of total salts)	Constituent acids (as percentage of glycine- or taurine-conjugated fraction)				
				Litho-cholic acid	Chenodeoxy-cholic acid	Deoxy-cholic acid	Cholic acid	3α:12α-Dihydroxy-7-oxocholanic acid
1	Man	1·07	Glycine 67	—	43	29	28	—
			Taurine 33	—	44	30	26	—
2	Man	0·80	Glycine 57	—	46	20	34	—
			Taurine 43	—	49	21	30	—
3	Man	0·68	Glycine 90	—	44	19	37	—
			Taurine 10	—	42	18	40	—
4	*Pan satyrus*	0·28	Glycine 60	—	21	14	65	—
			Taurine 40	—	16	11	73	—
5	*Cercopithecus mona*	1·53	Glycine 87	3	5	40	52	—
			Taurine 13	—	4	39	57	—
6	*C. aethiops tantalus*	0·44	Glycine 85	—	13	51	32	4
			Taurine 15	—	13	51	36	—
7	*C. nictitans mortinii*	0·15	Glycine 46	—	13	20	66	—
			Taurine 54	—	22	33	45	—
8	*C. nictitans mortinii*	0·42	Glycine 92	—	5	39	53	3
			Taurine 8	—	4	40	56	—

If the results presented here are combined with those previously reported for human-bile constituents (Wootton & Wiggins, 1953), it will be seen that, in every human bile analysed, chenodeoxycholic acid constitutes more than 30 % of the total bile acid. This relatively high proportion of chenodeoxycholic acid still seems to be characteristic of human bile.

SUMMARY

1. A series of eight primate-bile-salt specimens has been analysed. The method combined a countercurrent procedure, which separated glycine and taurine conjugates, and chromatography controlled by infrared spectroscopy.

2. Within experimental error, the glycine-conjugated and taurine-conjugated fractions contained the same bile acids in similar proportions.

3. The major constituent acids in all the bile samples were chenodeoxycholic acid (3α:7α-dihydroxycholanic acid), deoxycholic acid (3α:12α-dihydroxycholanic acid) and cholic acid (3α:7α:12α-trihydroxycholanic acid). Only in human bile does chenodeoxycholic acid occur as more than 30 % of the total acids.

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